Bayer CropScience GmbH

Plants with increased activity of multiple starch phosphorylating enzymes

Description

The present invention relates to plant cells and plants, which are genetically modified, wherein the genetic modification leads to an increase in the activity of a starch phosphorylating OK1 protein and a starch phosphorylating R1 protein in comparison with corresponding wild type plant cells or wild type plants that have not been genetically modified. Furthermore, the present invention relates to means and methods for the manufacture of such plant cells and plants. Plant cells and plants of this type synthesise a modified starch. The present invention therefore also relates to the starch synthesised by the plant cells and plants according to the invention, methods for the manufacture of this starch, and the manufacture of starch derivatives of this modified starch, as well as flours containing starches according to the invention.

Furthermore, the present invention relates to nucleic acid molecules and vectors containing sequences which code for an OK1 protein and an R1 protein, as well as host cells which contain these nucleic acid molecules.

With regard to the increasing importance currently attributed to plant constituents as renewable raw material sources, one of the tasks of biotechnological research is to endeavour to adapt these plant raw materials to suit the requirements of the processing industry. Furthermore, in order to enable regenerating raw materials to be used in as many areas of application as possible, it is necessary to achieve a large variety of materials.

The polysaccharide starch is made up of chemically uniform base components, the glucose molecules, but constitutes a complex mixture of different molecule forms, which exhibit differences with regard to the degree of polymerisation and branching, and therefore differ strongly from one another in their physical-chemical characteristics. Discrimination is made between amylose starch, an essentially unbranched polymer made from alpha-1,4-glycosidically linked glucose units, and the amylopectin starch, a branched polymer, in which the branches come about by the occurrence of additional alpha-1,6-glycosidic links. A further essential difference between amylose and amylopectin lies in the molecular weight. While amylose, depending on the origin of the starch, has a molecular weight of $5x10^5 - 10^6$ Da, that of the amylopectin lies between 10^7 and 10^8 Da. The two macromolecules can be differentiated by their molecular weight and their different physical-chemical characteristics, which can most easily be made visible by their different iodine bonding characteristics.

Amylose has long been looked upon as a linear polymer, consisting of alpha-1,4-glycosidically linked alpha-D-glucose monomers. In more recent studies, however, the presence of alpha-1,6-glycosidic branching points (ca. 0.1%) has been shown (Hizukuri and Takagi, Carbohydr. Res. 134, (1984), 1-10; Takeda et al., Carbohydr. Res. 132, (1984), 83-92).

The functional characteristics of the starch such as, for example, the solubility, the retrogradation behaviour, the water bonding capability, the film formation characteristics, the viscosity, the sticking characteristics, the freezing-thawing stability, the acid stability, the gelling strength, the starch granule size of the starches, and others are affected by the amylose/amylopectin ratio, the molecular weight, the pattern of the side chain distribution, the ion concentration, the lipid and protein content, the average starch granule size of the starch, the starch granule morphology, etc. The functional characteristics of starch are also affected by phosphate content, a non-carbon component of starch. Discrimination is made between phosphate which is covalently bound to the glucose molecules in the form of monoesters (designated

below as starch phosphate) and phosphate in the form of phospholipids with the starch associated.

The concentration of starch phosphate varies depending on plant type. Thus certain maize mutants synthesise a starch with an increased concentration of starch phosphate (waxy maize 0.002% and high-amylose maize 0.013%), while conventional maize species have only traces of starch phosphate. Small amounts of starch phosphate are likewise found in wheat (0.001%), while no starch phosphate has been shown in oats and sorghum. Likewise, less starch phosphate is found in rice mutants than in conventional rice species (0.013%). Significant amounts of starch phosphate have been shown in bulb or storage-root starch-synthesizing plants such as tapioca (0.008%), sweet potato (0.011%), arrowroot (0.021%) or potato (0.089%). The percentage values cited above for starch phosphate content relate to the respective dry weight of the starch and have been determined by Jane et al. (1996, Cereal Foods World 41 (11), 827-832).

Starch phosphate can exists in the form of monoesters at the C-2, C-3 or C-6 position of the polymerised glucose monomers (Takeda and Hizukuri, 1971, Starch/Stärke 23, 267-272). The phosphate distribution of the phosphate in starch synthesised by plants thus generally shows that approximately 30% to 40% of the phosphate residues are covalently bound in the C-3 position and approximately 60% to 70% of the phosphate residues are covalently bound in the C-6 position of the glucose molecules (Blennow et al., Int. J. of Biological Macromolecules 27, 211-218). Blennow et al. (2000, Carbohydrate Polymers 41, 163-174) determined a concentration of starch phosphate that is bound in the C-6 position of the glucose molecules for various starches such as potato starch (between 7.8 and 33.5 nMol per mg of starch, depending on species), starch from various *Curcuma* species (between 1.8 and 63 nMol per mg), tapioca starch (2.5 nMol per mg of starch), rice starch (1.0 nMol per mg of starch), mung bean starch (3.5 nMol per mg of starch) and sorghum starch (0.9 nMol per mg of starch). These authors could detect no bound starch phosphate in the C-6 position

in barley starch and starch from various waxy mutants of maize. No connection between the genotype of a plant and the concentration of starch phosphate has yet been established (Jane et al., 1996, Cereal Foods World 41 (11), 827-832). Therefore it is presently not possible to affect the concentration of starch phosphate in plants through breeding measures.

Only one protein which causes the introduction of covalent bonds of phosphate residues to the glucose molecules of starch was previously described. This protein has the enzymatic activity of an alpha-glucan water dikinase (GWD, E.C.: 2.7.9.4) (Ritte et al., 2002, PNAS 99, 7166-7171), is often designated as R1 in the scientific literature and is bound to the starch granules of the storage starch in potato tubers (Loberth et al., 1998, Nature Biotechnology 16, 473-477). In the reaction catalysed by R1, the educts alpha-1,4-glucan (starch), adenosine triphosphate (ATP) and water are converted to the products glucan phosphate (starch phosphate), monophosphate and adenosine monophosphate. At the same time, the gamma phosphate residue of the ATP is transferred to water, and the beta phosphate residue of the ATP is transferred to the glucan (starch). R1 transfers the beta-phosphate residue *in vitro* from ATP to the C-6- and the C-3 position of the glucose molecules of alpha-1,4-glucans. The ratio of C-6 phosphate to C-3 phosphate which is obtained through the *in vitro* reaction corresponds to the ratio which exists in starch isolated from plants (Ritte et al., 2002, PNAS 99, 7166-7171). As about 70% of the starch phosphate present in potato starch is bonded to the glucose monomers of starch in the C-6 position and about 30% in the C-3 position, this means that R1 preferably phosphorylates the C-6 position of the glucose molecules. Furthermore, it has been shown that by the use of amylopectin from maize, among other things, R1 can phosphorylate alpha-1,4-glucans, which do not yet contain covalently bonded phosphate (Ritte et al., 2002, PNAS 99, 7166-7171), i.e. R1 is able to introduce phosphate *de novo* into alpha-1,4-glucans.

Wheat plants which have an increased activity of an R1 protein through overexpression of an R1 gene from potatoes are described in WO 02 34923. These plants synthesise a starch with significant amounts of starch phosphate in the C-6 position of the glucose molecules in comparison with corresponding wild type plants in which no starch phosphate could be detected.

Further proteins, which catalyse a reaction, which introduce covalently bound phosphate groups into the starch, have not been previously described. Enzymes, which preferably introduce phosphate groups in the C-3 position and/or the C-2 position of the glucose molecules of starch, are also unknown. Apart from the increase of the starch phosphate content in plants, there are therefore also no available ways of specifically influencing the phosphorylation of starch in plants, of modifying the phosphate distribution within the starch synthesised by plants, and/or of further increasing the starch phosphate content.

The object of the present invention is therefore based on providing modified starches with increased phosphate content and/or changed phosphate distribution as well as plant cells and/or plants that synthesise such a modified starch, as well as methods and means for the production of said plants and/or plant cells.

This problem is solved by the embodiments described in the claims.

The present invention therefore relates to genetically modified plant cells and plants, characterised in that they have an increased activity of at least one OK1 protein and at least one R1 protein in comparison with corresponding wild type plant cells or wild type plants that have not been genetically modified.

A first aspect of the present invention relates to a plant cell or plant, which is genetically modified, wherein the genetic modification leads to an increase in the activity of at least one OK1 protein and, simultaneously, at least one R1 protein, in

comparison with corresponding wild type plant cells or wild type plants that have not been genetically modified.

At the same time, the genetic modification can be any genetic modification, which leads to an increase in the activity of at least one OK1 protein and (simultaneously) at least one R1 protein in genetically modified plant cells or genetically modified plants in comparison with corresponding wild type plant cells or wild type plants that have not been genetically modified.

In conjunction with the present invention, the term "wild type plant cell" means that the plant cells concerned were used as starting material for the manufacture of the plant cells according to the invention, i.e. their genetic information, apart from the introduced genetic modification, corresponds to that of a plant cell according to the invention.

In conjunction with the present invention, the term "wild type plant" means that the plants concerned were used as starting material for the manufacture of the plants according to the invention, i.e. their genetic information, apart from the introduced genetic modification, corresponds to that of a plant according to the invention.

In conjunction with the present invention, the term "corresponding" means that, in the comparison of several objects, the objects concerned that are compared with one another have been kept under the same conditions. In conjunction with the present invention, the term "corresponding" in conjunction with wild type plant cell or wild type plant compared to genetically modified plant cells or plants means that the plant cells or plants, which are compared with one another, have been raised under the same cultivation conditions and that they have the same (cultivation) age.

Here, within the framework of the present invention, the term "increased activity of at least one OK1 protein" means an increase in the expression of endogenous genes,

which code OK1 proteins and/or an increase in the quantity of OK1 protein in the cells and/or an increase in the enzymatic activity of OK1 proteins in the cells.

Here, within the framework of the present invention, the term "increased activity of at least one R1 protein" means an increase in the expression of endogenous genes, which code R1 proteins and/or an increase in the quantity of R1 protein in the cells and/or an increase in the enzymatic activity of R1 proteins in the cells.

The increase in the expression can, for example, be determined by measuring the quantity of transcripts coding OK1 proteins or R1 proteins. This can take place through Northern blot analysis or RT-PCR. Here, an increase preferably means an increase in the quantity of transcripts in comparison with corresponding cells that have not been genetically modified by at least 50%, in particular by at least 70%, preferably by at least 85% and particularly preferably by at least 100%. An increase in the quantity of transcripts coding an OK1 protein also means that plants or plant cells that have no detectable quantities of transcripts coding an OK1 protein have detectable quantities coding an OK1 protein after genetic modification according to the invention. An increase in the quantity of transcripts coding an R1 protein also means that plants or plant cells that have no detectable quantities of transcripts coding an R1 protein have detectable quantities of transcripts coding an R1 protein after genetic modification according to the invention.

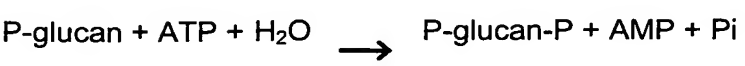
The increase in the amount of protein of an OK1 protein or an R1 protein, which results in an increased activity of these proteins in the plant cells concerned, can, for example, be determined by immunological methods such as Western blot analysis, ELISA (Enzyme Linked Immuno Sorbent Assay) or RIA (Radio Immune Assay). Here, an increase preferably means an increase in the amount of protein in comparison with corresponding cells that have not been genetically modified by at least 50%, in particular by at least 70%, preferably by at least 85% and particularly preferably by at least 100%. An increase in the amount of an OK1 protein also means that plants or

plant cells that have no detectable activity of an OK1 protein have a detectable amount of an OK1 protein after genetic modification according to the invention. An increase in the amount of an R1 protein also means that plants or plant cells that have no detectable activity of an R1 protein have a detectable amount of an R1 protein after genetic modification according to the invention.

Methods for the manufacture of antibodies that react specifically with a designated protein, i.e. that bind specifically to the said protein, are known to the person skilled in the art (see, for example, Lottspeich and Zorbas (Eds.), 1998, Bioanalytik, Spektrum akad, Verlag, Heidelberg, Berlin, ISBN 3-8274-0041-4). The manufacture of such antibodies is offered as a contractual service by several firms (for example, Eurogentec, Belgium). A possibility for manufacture of antibodies that react specifically with an OK1 protein is further described below (see Example 10). An antibody with which an increase in the amount of R1 protein can be determined by means of immunological methods is described by Lorberth et al. (1998, Nature Biotechnology 16, 473-477) and Ritte et al. (2000, Plant Journal 21, 387-391).

Within the framework of the present invention, the term "OK 1 protein" should be understood to mean a protein that transfers a phosphate residue from ATP to starch that is already phosphorylated (P-starch). Starches isolated from leaves of an *Arabisopsis thaliana sex1-3* mutant have no detectable quantity of covalently bound phosphate residues and are not phosphorylated by an OK1 protein, i.e., an OK1 protein according to the invention requires starch that is already phosphorylated as a substrate for the transfer of additional phosphate residues.
Preferably, the beta phosphate residue of the ATP is transferred from an OK1 protein to the starch and the gamma phosphate residue of the ATP is transferred to water. As an additional reaction product, AMP (adenosine monophosphate) is formed during a phosphorylation reaction of P-starch carried out by an OK1 protein. An OK1 protein is therefore designated as [phosphorylated alpha-glucan]-water-dikinase ([P-glucan]-water-dikinase) or as [phosphorylated starch] water-dikinase.

Therefore, OK1 proteins catalyse a reaction according to the following formula:



Preferably, an additional phosphate monoester bond in the C-6 position and/or in the C-3 position of a glucose molecule of the P-starch is formed on the P-starch phosphorylated by an OK1 protein. Particularly preferably, several additional phosphate monoester bonds in the C-3 position are formed during the phosphorylation of P-starch catalysed by an OK1 protein in comparison with phosphate monoester bonds in the C-6 position of the glucose molecules of the corresponding P-starch.

Amino acid sequences that code OK1 proteins contain a phosphohistidine domain. Phosphohistidine domains are, for example, described by Tien-Shin Yu et al. (2001, Plant Cell 13, 1907-1918). Phosphohistidine domains from OK1 proteins coding amino acid sequences preferably contain two histidines.

During the catalysis of a phosphorylation reaction of P-starch through an OK1 protein, a phosphorylated OK1 protein is formed as an intermediate product, through which a phosphate residue of the ATP is covalently bound to an amino acid of the OK1 protein. The intermediate product is formed through autophosphorylation of the OK1 protein, i.e., the OK1 protein itself catalyses the reaction that leads to the intermediate product. Preferably, a histidine residue of the amino acid sequence coding an OK1 protein is phosphorylated through the autophosphorylation, particularly preferably a histidine residue that is part of a phosphohistidine domain.

Furthermore, OK1 proteins according to the invention have an increased bonding activity to P-starch in comparison with non-phosphorylated starch.

Because no enzymes have yet been described that require P-starch as a substrate in order to phosphorylate them further, it was not possible until now to increase the concentration of starch phosphate of starch that is already phosphorylated in plants beyond a certain quantity. This is only possible through the use of a protein according to the invention or a nucleic acid molecule according to the invention for genetic

modification of plants. The clarification of the function of an OK1 protein and with it the preparation of an OK1 protein means that only plants that synthesise a starch with modified characteristics can be genetically modified to that effect. The modification of the phosphate distribution in starch synthesised by plants was not possible until now because of the lack of available means. Through the preparation of proteins according to the invention and nucleic acids through the present invention, a modification of the phosphate ratio in native starches is now possible as well. A further advantage of the present invention is that, for a simultaneous cooperation of an OK1 protein with an R1 protein, higher amounts of phosphate are incorporated into the starch than when the respective proteins separated from one another in space or time phosphorylate starch or P-starch, respectively.

Within the framework of the present invention, the term "R1 protein" should be understood to mean a protein that transfers a phosphate residue from ATP to starch. Starches isolated from leaves of an *Arabidopsis thaliana sex1-3* mutant have no detectable amount of covalently bound phosphate residues but are phosphorylated from an R1 protein. This means non-phosphorylated starch, for example, isolated from leave of an *Arabidopsis thaliana sex1-3* mutant, is used as a substrate in a phosphorylation reaction catalysed by an R1 protein.

Preferably, the beta phosphate residue of the ATP is transferred from an R1 protein to the starch and the gamma phosphate residue of the ATP is transferred to water. AMP (adenosine monophosphate) results as an additional reaction product. An R1 protein is therefore designated as [alpha-1,4-glucan]-water-dikinase or as starch-water-dikinase (E.C.: 2.7.9.4; Ritte et al., 2002, PNAS 99, 7166-7171).

During the phosphorylation of starch catalysed by an R1 protein, more additional phosphate monoester bonds result in the C-6 position of the glucose molecules in comparison with phosphate monoester bonds in the C-3 position of the glucose molecules of the respective starch. By a R1 protein, approximately 60% to 70% of the phosphate residue is introduced in the C-6 position of the glucose molecules of starch, and approximately 30% to 40% of the phosphate residue is introduced in the

C-3 position of the glucose molecules of starch (Ritte et al., 2002, PNAS 99, 7166-7171).

During the catalysis of a phosphorylation reaction of starch through an R1 protein, a phosphorylated R1 protein results as an intermediate product, through which a phosphate residue of the ATP is covalently bound to an amino acid of the R1 protein (Ritte et al., 2002, PNAS 99, 7166-7171). The intermediate product results through autophosphorylation of the R1 protein, i.e., the R1 protein itself catalyses the reaction that leads to the intermediate product. Amino acid sequences that code R1 proteins contain a phosphohistidine domain. Phosphohistidine domains are, for example, described by Tien-Shin Yu et al. (2001, Plant Cell 13, 1907-1918). Phosphohistidine domains from R1 proteins coding amino acid sequences preferably contain one histidine. Through the autophosphorylation of an R1 protein, a histidine residue in the phosphohistidine domain of the amino acid sequence coding an R1 protein is phosphorylated (Mikkelsen et al., 2003, Biochemical Journal Intermediate Publication). Published on October 2003 as manuscript BJ20030999; Mikkelsen et al., 2004, Biochemical Journal 377, 525-532).

Nucleic acid sequences and corresponding amino acid sequences coding an R1 protein are described from different species such as potato (WO 97 11188, GenBank Acc.: AY027522, Y09533), wheat (WO 00 77229, US 6,462,256, GenBank Acc.: AAN93923, GenBank Acc.: AR236165), rice (GenBank Acc.: AAR61445, GenBank Acc.: AR400814), maize (GenBank Acc.: AAR61444, GenBank Acc.: AR400813), soybean (GenBank Acc.: AAR61446, GenBank Acc.: AR400815), citrus (GenBank Acc.: AY094062) and *Arabidopsis* (GenBank Acc.: AF312027). The identified nucleic acid sequences and amino acid sequences coding R1 proteins are published by NCBI (http://www.ncbi.nlm.nih.gov/entrez/), among others, and are explicitly included in the description of the present application by mention of the references.

In conjunction with the present invention, the term "increased bonding activity" is to be understood as an increased affinity of a protein for a first substrate in comparison to a second substrate. That is to say, the amount of protein, which, under the same

incubation conditions, bonds to a first substrate to a greater extent in comparison with a second substrate, exhibits increased bonding activity to the first substrate.

In conjunction with the present invention, the term "starch phosphate" is to be understood as phosphate groups covalently bound to the glucose molecules of starch.

In conjunction with the present invention, the term "non-phosphorylated starch" is to be understood as a starch that contains no detectable amount of starch phosphate. Various methods are described for the determination of the amount of starch phosphate. Preferably, the methods described by Ritte et al. (2000, Starch/Stärke 52, 179-185) can be used for the determination of the amount of starch phosphate. Particularly preferably, the determination of the amount of starch phosphate is carried out by means of ^{31}P-NMR according to the methods described by Kasemusuwan and Jane (1996, Cereal Chemistry 73, 702-707).

In conjunction with the present invention, the term "phosphorylated starch" or "P-starch" is to be understood as a starch that contains starch phosphate.

The activity of an OK1 protein can be shown, for example, through *in vitro* incubation of an OK1 protein using ATP that contains a labeled phosphate residue (labeled ATP) in the beta position. Preferred is ATP for which the phosphate residue is specifically labeled in the beta position, i.e., for which only the phosphate residue in the beta position bears a label. Preferably used is radioactively labeled ATP. Particularly preferably used is ATP for which the phosphate residue is specifically radioactively labeled, and especially preferably used is ATP for which the phosphate residue is specifically labeled with ^{33}P in the beta position. If an OK1 protein with labeled ATP and starches that are not phosphorylated is incubated, no phosphate is transferred through OK1 to the starch. Preferably used is leaf starch of the *Arabidopsis thaliana* mutant *sex1-3* (Tien-Shin Yu et al., 2001, Plant Cell 13, 1907-1918).

However, if an OK1 protein with P-starch is incubated in the presence of labeled ATP, then labeled phosphate covalently bound to the P-starch can be subsequently shown. Preferably used is starch from leaves of *Arabidopsis thaliana,* particularly preferably by means of an enzymatically-phosphorylated starch of an R1 protein from *Arabidopsis thaliana sex1-3* mutant (Ritte et al., 2002, PNAS 99, 7166-7171).

Labeled phosphate residues can be shown that were assembled by an OK1 protein in P-starch, for example, through separation of the labeled P-starch (for example, through precipitation means ethanol, filtration, chromatographic methods, etc.) from the residue of the reaction mixture and subsequent detection of the labeled phosphate residue in the P-starch fraction. At the same time, the labeled phosphate residues bound in the P-starch fraction can be shown, for example, through determination of the amount of the radioactivity existing in the P-starch fraction (for example, by means of a scintillation counter). Possible methods for the detection of a protein, which P-starch as a substrate for a phosphorylation reaction makes necessary, is further described below under General Methods, Item 11 and in example 6.

The activity of an R1 protein can be shown as described in the literature, for example (Mikkelsen et al., 2003, Biochemical Journal Intermediate Publication. Published on October 2003 as manuscript BJ20030999; Mikkelsen et al., 2004, Biochemical Journal 377, 525-532, Ritte et al., 2002, PNAS 99, 7166-7171).

Which positions of the carbon atoms (C-2, C-3 or C-6) of the glucose monomers in P-starch are preferably phosphorylated by an OK1 protein can be determined, for example, by analysing the P-starches phosphorylated by a protein, as described by Ritte et al. (2002, PNAS 99, 7166-7171). For this purpose, a P-starch phosphorylated by a protein is hydrolysed using an acid, and subsequently analysed by means of anion exchange chromatography.

Preferably, the P-starch phosphorylated by an OK1 protein is analysed by means of NMR in order to establish which positions of the carbon atoms (C-2, C-3 or C-6) of

the glucose monomers in the P-starch are phosphorylated. A particularly preferred method for identifying the C-atom positions of a glucose molecule of a starch, which are phosphorylated by a reaction catalysed by an OK1 protein, is described below under General Methods, Item 13.

Which positions of the carbon atoms (C-2, C-3 or C-6) of the glucose monomers in starch are preferably phosphorylated by an R1 protein can be determined, for example, by analysing the starches phosphorylated by an R1 protein, as described by Ritte et al. (2002, PNAS 99, 7166-7171). For this purpose, a starch phosphorylated by a protein is hydrolysed using an acid, and subsequently analysed by means of anion exchange chromatography.

Preferably, the P-starch phosphorylated by an OK1 protein, or the starch phosphorylated by an R1 protein, is analysed by means of NMR in order to establish which positions of the carbon atoms (C-2, C-3 or C-6) of the glucose monomers in the P-starch or the starch are phosphorylated. A particularly preferred method for identifying the C-atom positions of a glucose molecule of a starch, which are phosphorylated by a reaction catalysed by an OK1 protein or an R1 protein, is described below under General Methods, Item 13.

A phosphorylated protein, which is produced as an intermediate product in the phosphorylation of P-starch facilitated by an OK1 protein, can be demonstrated for an R1 protein as described, for example, by Ritte et al. (2002, PNAS 99, 7166-7171) or Mikkelsen et al. (2003, Biochemical Journal Intermediate Publication. Published on October 2003 as manuscript BJ20030999, Mikkelsen et al., 2004, Biochemical Journal 377, 525-532)

To demonstrate the presence of an autophosphorylated intermediate product, an OK1 protein is first incubated in the absence of starch with labeled ATP, preferably with ATP specifically labeled in the beta phosphate position, particularly preferably with

ATP specifically labeled with ^{33}P in the beta phosphate position. In parallel with this, a reaction preparation 2, which instead of labeled ATP contains corresponding amounts of non- labeled ATP however, is incubated under otherwise identical conditions. Subsequently, non- labeled ATP is added to the reaction mixture 1 in excess, and a mixture of non- labeled ATP and labeled ATP (the same amount of labeled ATP as used previously in reaction mixture 1 and the same amount of non- labeled ATP as added to reaction mixture 1 in excess) is added to reaction mixture 2 and further incubated before P-starch is added to a Part A of reaction mixture 1 (Part 1A) or to a Part A of reaction mixture 2 (Part 2A) respectively. The reaction in the remaining Part 1B and Part 2B of the reaction mixture is stopped by denaturing the protein. Part B of the reaction mixture can be stopped by the methods known to the person skilled in the art, which lead to the denaturing of proteins, preferably by adding sodium lauryl sulphate (SDS). Part 1A and Part 2A of the reaction mixture are incubated for at least a further 10 minutes before these reactions are also stopped. The starch present in Part A and Part B of the respective reaction mixture is separated from the remainder of the reaction mixture. If the respective starch is separated by centrifugation, for example, then, on completion of centrifugation, the starch of the respective Part A or Part B of the reaction mixture is to be found in the sedimented pellet, and the proteins in the respective reaction mixture are to be found in the supernatant of the respective centrifugation. The supernatant of Part 1A or 2A respectively and Part 1B or 2B respectively of the reaction mixture can subsequently be analysed by denaturing acrylamide gel electrophoresis followed by autoradiography of the acrylamide gel obtained. To quantify the amount of radioactively labeled proteins, which have been separated by means of acrylamide gel electrophoresis, the so-called "phospho-imaging" method, for example, known to the person skilled in the art, can be used. If the autoradiography or the analysis by means of the "phospho-imager" of proteins in the centrifugation supernatant of Part B of reaction mixture 1 shows a significantly increased signal compared with the centrifugation supernatant of Part A of reaction mixture 1, then this shows that a protein facilitating a phosphorylation of starch occurs as an autophosphorylated intermediate product. Parts A and B of reaction mixture 2

serve as a control and should therefore not exhibit a significantly increased signal in the centrifugation supernatant in the autoradiography or in the analysis by means of the "phospho-imager".

In addition, the starch of the respective Part A of reaction mixtures 1 and 2 remaining in the respective sedimented pellet can be investigated, if necessary, after subsequent washing of the respective starches, for the presence of starch phosphate, which has a label corresponding to the labeled ATP used. If the starches of Part A of reaction mixture 1 contain labeled phosphate residues, and if the autoradiography of the centrifugation supernatant of Part B of reaction mixture 1 shows a significantly increased signal in the autoradiography compared with the centrifugation supernatant of Part A of reaction mixture 1, then this shows that a phosphorylation of starch-facilitating protein is present as an autophosphorylated intermediate product. Parts A and B of reaction mixture 2 serve as a control and should therefore not exhibit a significantly increased signal for alpha-1,4-glucans labeled with e.g. ^{33}P in the sedimented pellet containing alpha-1,4-glucans. Possible methods for demonstrating a phosphorylated OK1 protein intermediate product are described below under General Methods, Item 12 and in Example 7.

That an OK1 protein has an increased bonding activity to a P-starch compared with non-phosphorylated starch can be demonstrated by incubating the OK1 protein with P-starch and non-phosphorylated starch in separate preparations.

All non-phosphorylated starches are basically suitable for incubating OK1 proteins with non-phosphorylated starch. Preferably, a non-phosphorylated plant starch, particularly preferably wheat starch, and especially preferably granular leaf starch of an *Arabidopsis thaliana* mutant *sex1-3* is used.

Methods for isolating starch from plants, for example, are known to the person skilled in the art. All methods known to the person skilled in the art are basically suitable for isolating non-phosphorylated starch from appropriate plant species. Preferably, the methods for isolating non-phosphorylated starch described below are used (see General Methods Item 2).

All starches that contain starch phosphate are basically suitable for incubating OK1 proteins with P-starch. Chemically phosphorylated starches can also be used for this purpose. Preferably, P-starches are used for the incubation with OK1 proteins, particularly preferably a retrospectively enzymatically phosphorylated plant starch, especially preferably a retrospectively enzymatically phosphorylated plant starch, which has been isolated from a *sex-1* mutant of *Arabidopsis thaliana*.

To demonstrate an increased bonding activity of OK1 proteins to P-starch compared with non-phosphorylated starch, OK1 proteins are incubated in separate preparations with P-starch (Preparation A) and with non-phosphorylated starch (Preparation B). On completion of the incubation, the proteins, which are not bonded to the related starches of Preparations A and B, are separated from the starches and from the proteins bonded to them. The bond between the proteins and the P-starch in Preparation A and the bond between the proteins and non-phosphorylated starch in Preparation B are subsequently removed, i.e. the respective proteins are dissolved. The dissolved proteins of Preparation A and Preparation B can then be separated from the starches concerned, which are present in the respective preparations. Following this, the isolated P-starch bonding proteins of Preparation A and the isolated non-phosphorylated starch bonding proteins of Preparation B can be separated with the help of methods known to the person skilled in the art such as, for example, gel filtration, chromatographic methods, electrophoresis, SDS acrylamide gel electrophoresis etc. By comparing the amounts of separated proteins of Preparation A with the amounts of corresponding separated proteins of Preparation B, it can be determined whether a protein has an increased bonding activity with respect to P-starch compared with non-phosphorylated starch. Methods, which can be used to demonstrate a preferred bonding of proteins to P-starch compared with non-phosphorylated starch, are described below in Example 8.

The amino acid sequence shown in SEQ ID NO 2 codes an OK1 protein from *Arabidopsis thaliana* and the amino acid sequence shown under SEQ ID NO 4 codes an OK1 protein from *Oryza sativa*.

In a further embodiment of the present invention, amino acid sequences coding an OK1 protein have an identity of at least 60% with the sequence specified in SEQ ID NO 2 or SEQ ID NO 4, in particular of at least 70%, preferably of at least 80% and particularly preferably of at least 90% and especially preferably of at least 95%.

In a further embodiment of the present invention, the OK1 protein has a phosphohistidine domain. Amino acid sequences coding OK1 proteins contain a phosphohistidine domain that has an identity of at least 60% with the amino acid sequence of the phosphohistidine domain of the OK1 protein from *Arabidopsis thaliana* specified in SEQ ID NO 5, in particular of at least 70%, preferably of at least 80% and particularly preferably of at least 90% and especially preferably of at least 95%.

A further embodiment of the present invention relates to a genetically modified plant cell according to the invention or a genetically modified plant according to the invention, wherein the genetic modification consists in the introduction of at least one foreign nucleic acid molecule into the genome of the plant cell or into the genome of the plant.

In this context, the term "genetic modification" means the introduction of homologous and/or heterologous foreign nucleic acid molecules into the genome of a plant cell or into the genome of a plant, wherein said introduction of these molecules leads to an increase in the activity of an OK1 protein and to the increase of the activity of an R1 protein.

The plant cells according to the invention or plants according to the invention are modified with regard to their genetic information by the introduction of a foreign

nucleic acid molecule. The presence or the expression of a foreign nucleic acid molecule leads to a phenotypic change. Here, "phenotypic" change means preferably a measurable change of one or more functions of the cells. For example, the genetically modified plant cells according to the invention and the genetically modified plants according to the invention exhibit an increase of the activity of an OK1 protein and an increase of the activity of an R1 protein due to the presence or on the expression of the introduced nucleic acid molecule.

In conjunction with the present invention, the term "foreign nucleic acid molecule" is understood to mean such a molecule that either does not occur naturally in the corresponding wild type plant cells, or that does not occur naturally in the concrete spatial arrangement in wild type plant cells, or that is localised at a place in the genome of the wild type plant cell at which it does not occur naturally. Preferably, the foreign nucleic acid molecule is a recombinant molecule, which consists of different elements, the combination or specific spatial arrangement of which does not occur naturally in plant cells.
In principle, a foreign nucleic acid molecule can be any nucleic acid molecule that effects an increase in the activity of an OK1 protein and an R1 protein in the plant cell or plant.

In conjunction with the present invention, the term "genome" is to be understood to mean the totality of the genetic material present in a plant cell. It is known to the person skilled in the art that, as well as the cell nucleus, other compartments (e.g. plastids, mitochondrions) also contain genetic material.

In a further embodiment, the plant cells according to the invention and the plants according to the invention are characterised in that at least one foreign nucleic acid molecule codes an OK1 protein, preferably an OK1 protein from *Arabidopsis thaliana* or an OK1 protein from *Oryza sativa*.

In a further embodiment, the foreign nucleic acid molecule codes an OK1 protein with the amino acid sequence specified in SEQ ID NO 2 or SEQ ID NO 4.

In a further embodiment, the plant cells according to the invention and the plants according to the invention are characterised in that at least one foreign nucleic acid molecule codes an R1 protein, preferably an R1 protein from potato or an OK1 protein from *Oryza sativa*.

In a further embodiment, the foreign nucleic acid molecule codes an R1 protein from potato with the amino acid sequence specified in GenBank Acc.: Y09533 (22-JUL-2003 Rel. 76, Last updated, Version 2). The nucleic acid molecules and amino acid sequences coding an R1 protein from potato (GenBank Acc.: Y09533) are explicitly included in the description of the present application by mention of the references.

In a further embodiment, the plant cells according to the invention and the plants according to the invention are characterised in that a first foreign nucleic acid molecule codes an R1 protein and a second foreign nucleic acid molecule codes an OK1 protein.

The foreign nucleic acid molecules assembled for the genetic modification in the plant cell or plant can be a single nucleic acid molecule or multiple nucleic acid molecules. It can therefore be nucleic acid molecules that contain nucleic acid sequences that code OK1 proteins and nucleic acid sequences that code R1 proteins, as well as nucleic acid molecules for which the nucleic acid sequences coding OK1 proteins and the nucleic acid sequences coding R1 proteins occur in various nucleic acid molecules. The nucleic acid sequences coding an OK1 protein and the nucleic acid sequences coding an R1 protein can be contained simultaneously, for example, in a vector, plasmid or linear nucleic acid molecule, or be, however, constituents of two vectors, plasmids or linear nucleic acid molecules respectively separated from one another.

If the nucleic acid sequences coding an OK1 protein and the nucleic acid sequences coding an R1 protein occur in two nucleic acid molecules separated from one another, then they can be introduced either simultaneously ("cotransformation") or also successively, i.e., consecutively ("supertransformation") into the genome of the plant cells or plant. The nucleic acid molecules separated from one another can also be introduced into various individual plant cells or plants of a species. Plant cells or plants can thereby be produced in which the activity of either at least one OK1 protein or at least one R1 protein is increased. Such plants can be produced by subsequently crossing the plants in which the activity of an OK1 protein is increased with plants in which the activity of an R1 protein is increased.

Furthermore, a mutant cell or a mutant that is characterised in that it already has an increased activity of an OK1 protein or an increased activity of an R1 protein can be used instead of a wild type plant cell or wild type plant for introducing a foreign nucleic acid molecule. Mutants can be spontaneously (naturally) occurring mutants as well as those that were produced through the targeted use of mutagens (such as, for example, chemical agents, ionising radiation) or genetic engineering methods (for example T-DNA activation tagging, transposon activation tagging, *in situ* activation, *in vivo* mutagenesis).

Therefore, plant cells according to the invention and plants according to the invention can also be produced through the introduction of a foreign nucleic acid molecule, which leads to the increase of the activity of an R1 protein in a mutant cell or a mutant that already has an increased activity of an OK1 protein. Plant cells according to the invention or plants according to the invention can also be produced through the introduction of a foreign nucleic acid molecule, which leads to the increase of the activity of an OK1 protein in a mutant cell or a mutant that already has an increased activity of an R1 protein.

Plant cells according to the invention or plants according to the invention can also be produced in which a mutant in which the activity of an OK1 protein is increased is crossed with a plant that has an increased activity of an R1 protein due to the

introduction of a foreign nucleic acid molecule. Likewise, it is possible to produce plant cells according to the invention or plants according to the invention in which a mutant in which the activity of an R1 protein is increased is crossed with a plant that has an increased activity of an OK1 protein due to the introduction of a foreign nucleic acid molecule.

A large number of techniques are available for the introduction of DNA into a plant host cell. These techniques include the transformation of plant cells with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as the transformation medium, the fusion of protoplasts, injection, the electroporation of DNA, the introduction of DNA by means of the biolistic approach as well as other possibilities. The use of agrobacteria-mediated transformation of plant cells has been intensively investigated and adequately described in EP 120516; Hoekema, IN: The Binary Plant Vector System Offsetdrukkerij Kanters B.V., Alblasserdam (1985), Chapter V; Fraley et al., Crit. Rev. Plant Sci. 4, 1-46 and by An et al. EMBO J. 4, (1985), 277-287. For the transformation of potato, see Rocha-Sosa et al., EMBO J. 8, (1989), 29-33, for example.

The transformation of monocotyledonous plants by means of vectors based on *Agrobacterium* transformation has also been described (Chan et al., Plant Mol. Biol. 22, (1993), 491-506; Hiei et al., Plant J. 6, (1994) 271-282; Deng et al., Science in China 33, (1990), 28-34; Wilmink et al., Plant Cell Reports 11, (1992), 76-80; May et al., Bio/Technology 13, (1995), 486-492; Conner and Domisse, Int. J. Plant Sci. 153 (1992), 550-555; Ritchie et al., Transgenic Res. 2, (1993), 252-265). An alternative system to the transformation of monocotyledonous plants is transformation by means of the biolistic approach (Wan and Lemaux, Plant Physiol. 104, (1994), 37-48; Vasil et al., Bio/Technology 11 (1993), 1553-1558; Ritala et al., Plant Mol. Biol. 24, (1994), 317-325; Spencer et al., Theor. Appl. Genet. 79, (1990), 625-631), protoplast transformation, electroporation of partially permeabilised cells and the introduction of DNA by means of glass fibres. In particular, the transformation of maize has been

described in the literature many times (cf. e.g. WO95/06128, EP0513849, EP0465875, EP0292435; Fromm et al., Biotechnology 8, (1990), 833-844; Gordon-Kamm et al., Plant Cell 2, (1990), 603-618; Koziel et al., Biotechnology 11 (1993), 194-200; Moroc et al., Theor. Appl. Genet. 80, (1990), 721-726).

The successful transformation of other types of cereal has also already been described, for example for barley (Wan and Lemaux, see above; Ritala et al., see above; Krens et al., Nature 296, (1982), 72-74) and for wheat (Nehra et al., Plant J. 5, (1994), 285-297; Becker et al., 1994, Plant Journal 5, 299-307). All the above methods are suitable within the framework of the present invention.

Amongst other things, plant cells and plants, which have been genetically modified by the introduction of an OK1 protein and/or an R1 protein, can be differentiated from wild type plant cells and wild type plants respectively in that they contain a foreign nucleic acid molecule, which does not occur naturally in wild type plant cells or wild type plants, or in that such a molecule is present integrated at a place in the genome of the plant cell according to the invention or in the genome of the plant according to the invention at which it does not occur in wild type plant cells or wild type plants, i.e. in a different genomic environment. Furthermore, plant cells according to the invention and plants according to the invention of this type differ from wild type plant cells and wild type plants respectively in that they contain at least one copy of the foreign nucleic acid molecule stably integrated within their genome, possibly in addition to naturally occurring copies of such a molecule in the wild type plant cells or wild type plants. If the foreign nucleic acid molecule(s) introduced into the plant cells according to the invention or into the plants according to the invention is (are) additional copies of molecules already occurring naturally in the wild type plant cells or wild type plants respectively, then the plant cells according to the invention and the plants according to the invention can be differentiated from wild type plant cells or wild type plants respectively in particular in that this additional copy or these additional copies is (are) localised at places in the genome at which it does not occur

(or they do not occur) in wild type plant cells or wild type plants. This can be verified, for example, with the help of a Southern blot analysis.

Furthermore, the plant cells according to the invention and the plants according to the invention can preferably be differentiated from wild type plant cells or wild type plants respectively by at least one of the following characteristics: If a foreign nucleic acid molecule that has been introduced is heterologous with respect to the plant cell or plant, then the plant cells according to the invention or plants according to the invention have transcripts of the introduced nucleic acid molecules. These can be verified, for example, by Northern blot analysis or by RT-PCR (Reverse Transcription Polymerase Chain Reaction). Plant cells according to the invention and plants according to the invention, which express an antisense and/or an RNAi transcript, can be verified, for example, with the help of specific nucleic acid probes, which are complimentary to the RNA (occurring naturally in the plant cell), which is coding for the protein. Preferably, the plant cells according to the invention and the plants according to the invention contain a protein, which is coded by an introduced nucleic acid molecule. This can be demonstrated by immunological methods, for example, in particular by a Western blot analysis.

If the foreign nucleic acid molecule that has been introduced is homologous with respect to the plant cell or plant, the plant cells according to the invention or plants according to the invention can be differentiated from wild type plant cells or wild type plants respectively due to the additional expression of the introduced foreign nucleic acid molecule, for example. The plant cells according to the invention and the plants according to the invention preferably contain transcripts of the foreign nucleic acid molecules. This can be demonstrated by Northern blot analysis, for example, or with the help of so-called quantitative PCR.

In a further embodiment, the plant cells according to the invention and the plants according to the invention are transgenic plant cells or transgenic plants respectively.

In a further embodiment, the present invention relates to plant cells according to the invention and plants according to the invention wherein the foreign nucleic acid molecule coding an OK1 protein is chosen from the group consisting of

a) Nucleic acid molecules, which code a protein with the amino acid sequence given under SEQ ID NO 2 or SEQ ID NO 4;

b) Nucleic acid molecules, which code a protein, which includes the amino acid sequence, which is coded by the insertion in plasmid A.t.-OK1-pGEM or the insertion in plasmid pMI50;

c) Nucleic acid molecules, which code a protein, the sequence of which has an identity of at least 60% with the amino acid sequence given under SEQ ID NO 2 or SEQ ID NO 4;

d) Nucleic acid molecules, which code a protein, the sequence of which has an identity of at least 60% with the amino acid sequence, which is coded by the coding region of the insertion in plasmid A.t.-OK1-pGEM or by the coding region of the insertion in plasmid pMI50;

e) Nucleic acid molecules, which include the nucleotide sequence shown under SEQ ID NO 1 or SEQ ID NO 3 or a complimentary sequence;

f) Nucleic acid molecules, which include the nucleotide sequence of the insertion contained in plasmid A.t.-OK1-pGEM or plasmid pMI50;

g) Nucleic acid molecules, which have an identity of at least 70% with the nucleic acid sequences described under a), b), e) or e);

h) Nucleic acid molecules, which hybridise with at least one strand of the nucleic acid molecules described under a), b), e) or f) under stringent conditions;

i) Nucleic acid molecules, the nucleotide sequence of which deviates from the sequence of the nucleic acid molecules identified under a), b), e) or f) due to the degeneration of the genetic code; and

j) Nucleic acid molecules, which represent fragments, allelic variants and/or derivatives of the nucleic acid molecules identified under a), b), c), d), e), f), g), h) or i).

The nucleic acid sequence shown SEQ ID NO 1 is a cDNA sequence, which includes the coding region for an OK1 protein from *Arabidopsis thaliana* and the nucleic acid sequence shown SEQ ID NO 3 is a cDNA sequence, which includes the coding region for an OK1 protein from *Oryza sativa*.

A plasmid (A.t.-OK1-pGEM) containing a cDNA which codes for a protein according to the invention (A.t.-OK1) from *Arabidopsis thaliana* was deposited on 08.03.2004 under the number DSM16264 and a plasmid (pM150) containing a cDNA which codes for further protein according to the invention (O.s.-OK1) from *Oryza sativa* was deposited on 24.03.2004 under the number DSM16302 under the Budapest Treaty at the German Collection of Microorganisms and Cell Cultures GmbH, Mascheroder Weg 1b, 38124 Braunschweig, Germany.

The amino acid sequence shown under SEQ ID NO 2 can be derived from the coding region of the cDNA sequence integrated in plasmid A.t.-OK1-pGEM and codes for an OK1 protein from *Arabidopsis thaliana*. The amino acid sequence shown SEQ ID NO 4 can be derived from the coding region of the cDNA sequence integrated in plasmid pMI50 and codes for an OK1 protein from *Oryza sativa*. The present invention therefore also relates to nucleic acid molecules, which code a protein with the enzymatic activity of an OK1 protein, which includes the amino acid sequence, which is coded by the insertion in plasmid A.t.-OK1-pGEM or by the insertion in plasmid pMI50, wherein the coded protein has an identity of at least 70%, preferably of at least 80%, particularly preferably of at least 90% and especially preferably of 95% with the amino acid sequence, which can be derived from the insertion in A.t.-OK1-pGEM or pMI50.

The present invention also relates to nucleic acid molecules, which code an OK1 protein and include the coding region of the nucleotide sequences shown under SEQ ID NO 1 or SEQ ID NO 3 or sequences, which are complimentary thereto, nucleic acid molecules, which include the coding region of the nucleotide sequence of the insertion contained in plasmid A.t.-OK1-pGEM or in plasmid pMI50 and nucleic acid molecules, which have an identity of at least 70%, preferably of at least 80%,

particularly preferably of at least 90% and especially preferably of at least 95% with the said nucleic acid molecules.

With the help of the sequence information of nucleic acid molecules according to the invention or with the help of a nucleic acid molecule according to the invention, it is now possible for the person skilled in the art to isolate homologous sequences from other plant species, preferably from starch-storing plants, preferably from plant species of the genus *Oryza*, in particular *Oryza sativa* or from *Arabidopsis thaliana*. This can be carried out, for example, with the help of conventional methods such as the examination of cDNA or genomic libraries with suitable hybridisation samples. The person skilled in the art knows that homologous sequences can also be isolated with the help of (degenerated) oligonucleotides and the use of PCR-based methods. The examination of databases, such as are made available, for example, by EMBL (http://www.ebi.ac.uk/Tools/index.htm) or NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/), can also be used for identifying homologous sequences, which code for OK1 proteins. In this case, one or more sequences are specified as a so-called query. This query sequence is then compared by means of statistical computer programs with sequences, which are contained in the selected databases. Such database queries (e.g. blast or fasta searches) are known to the person skilled in the art and can be carried out by various providers.
If such a database query is carried out, e.g. at the NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/), then the standard settings, which are specified for the particular comparison inquiry, should be used. For protein sequence comparisons (blastp), these are the following settings: Limit entrez = not activated; Filter = low compexity activated; Expect value = 10; word size = 3; Matrix = BLOSUM62; Gap costs: Existence = 11, Extension = 1.
For nucleic acid sequence comparisons (blastn), the following parameters must be set: Limit entrez = not activated; Filter = low compexity activated; Expect value = 10; word size = 11.

With such a database search, the sequences described in the present invention can be used as a query sequence in order to identify further nucleic acid molecules and/or proteins, which code an OK1 protein.

With the help of the described methods, it is also possible to identify and/or isolate nucleic acid molecules according to the invention, which hybridise with the sequence specified under SEQ ID NO 1 or under SEQ ID NO 3 and which code an OK1 protein. Within the framework of the present invention, the term "hybridising" means hybridisation under conventional hybridisation conditions, preferably under stringent conditions such as, for example, are described in Sambrock et al., Molecular Cloning, A Laboratory Manual, 2nd edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). Particularly preferably, "hybridising" means hybridisation under the following conditions:

Hybridisation buffer:

2xSSC; 10xDenhardt solution (Ficoll 400+PEG+BSA; Ratio 1:1:1); 0.1% SDS; 5 mM EDTA; 50 mM Na2HPO4; 250 µg/ml herring sperm DNA; 50 µg/ml tRNA; or

25 M sodium phosphate buffer pH 7.2; 1 mM EDTA; 7% SDS

Hybridisation temperature:

T=65 to 68°C

Wash buffer: 0.1xSSC; 0.1% SDS

Wash temperature: T=65 to 68°C.

In principle, nucleic acid molecules, which hybridise with the nucleic acid molecules according to the invention, can originate from any plant species, which codes an appropriate protein; preferably they originate from starch-storing plants, preferably from species of the (systematic) family *Poacea*, particularly preferably from species of the genus *Oryza*. Nucleic acid molecules, which hybridise with the molecules according to the invention, can, for example, be isolated from genomic or from cDNA libraries. The identification and isolation of nucleic acid molecules of this type can be carried out using the nucleic acid molecules according to the invention or parts of these molecules or the reverse complements of these molecules, e.g. by means of

hybridisation according to standard methods (see, for example, Sambrook et al., Molecular Cloning, A Laboratory Manual, 2nd edition Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY) or by amplification using PCR.

Nucleic acid molecules, which exactly or essentially have the nucleotide sequence specified under SEQ ID NO 1 or SEQ ID NO 3 or parts of these sequences, can be used as hybridisation samples. The fragments used as hybridisation samples can also be synthetic fragments or oligonucleotides, which have been manufactured using established synthesising techniques and the sequence of which corresponds essentially with that of a nucleic acid molecule according to the invention. If genes have been identified and isolated, which hybridise with the nucleic acid sequences according to the invention, then a determination of this sequence and an analysis of the characteristics of the proteins coded by this sequence should be carried out in order to establish whether an OK1 protein is involved. Homology comparisons on the level of the nucleic acid or amino acid sequence and a determination of the enzymatic activity are particularly suitable for this purpose. The activity of an OK1 protein can take place, for example, as described above under General Methods Item 11. A preferred bonding affinity to P-starch in comparison with non-phosphorylated starch, and autophosphorylation of an OK1 protein can be demonstrated using the methods already described above and under General Methods Items 8 and 12.

The molecules hybridising with the nucleic acid molecules according to the invention particularly include fragments, derivatives and allelic variants of the nucleic acid molecules according to the invention, which code an OK1 protein from plants, preferably from starch-storing plants, preferably from plant species of the (systematic) family *Poacea*, particularly preferably from species of the genus *Oryza*. In conjunction with the present invention, the term "derivative" means that the sequences of these molecules differ at one or more positions from the sequences of the nucleic acid molecules described above and have a high degree of identity with these sequences. Here, the deviation from the nucleic acid molecules described above can have come about, for example, due to deletion, addition, substitution, insertion or recombination.

In conjunction with the present invention, the term "identity" means a sequence identity over the whole length of the coding region of at least 60%, in particular an identity of at least 80%, preferably greater than 80%, particularly preferably greater than 90% and especially of at least 95%. In conjunction with the present invention, the term "identity" is to be understood to mean the number of amino acids/nucleotides (identity) corresponding with other proteins/nucleic acids, expressed as a percentage. Identity is preferably determined by comparing with other proteins/nucleic acids the SEQ ID NO 2 or SEQ ID NO 4 for amino acids or SEQ ID NO 1 or SEQ ID NO 3 for nucleic acids with the help of computer programs. If sequences that are compared with one another have different lengths, the identity is to be determined in such a way that the number of amino acids, which have the shorter sequence in common with the longer sequence, determines the percentage quotient of the identity. Preferably, identity is determined by means of the computer program ClustalW, which is well-known and available to the public (Thompson et al., Nucleic Acids Research 22 (1994), 4673-4680). ClustalW is made publicly available by Julie Thompson (Thompson@EMBL-Heidelberg.DE) and Toby Gibson (Gibson@EMBL-Heidelberg.DE), European Molecular Biology Laboratory, Meyerhofstrasse 1, D 69117 Heidelberg, Germany. ClustalW can also be downloaded from different Internet sites, including the IGBMC (Institut de Génétique et de Biologie Moléculaire et Cellulaire, B.P.163, 67404 Illkirch Cedex, France; ftp://ftp-igbmc.u-strasbg.fr/pub/) and the EBI (ftp://ftp.ebi.ac.uk/pub/software/) as well as from all mirrored Internet sites of the EBI (European Bioinformatics Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SD, UK).

Preferably, Version 1.8 of the ClustalW computer program is used to determine the identity between proteins according to the invention and other proteins. In doing so, the following parameters must be set: KTUPLE=1, TOPDIAG=5, WINDOW=5, PAIRGAP=3, GAPOPEN=10, GAPEXTEND=0.05, GAPDIST=8, MAXDIV=40, MATRIX=GONNET, ENDGAPS(OFF), NOPGAP, NOHGAP.

Preferably, Version 1.8 of the ClustalW computer program is used to determine the identity between the nucleotide sequence of the nucleic acid molecules according to

the invention, for example, and the nucleotide sequence of other nucleic acid molecules. In doing so, the following parameters must be set:
KTUPLE=2, TOPDIAGS=4, PAIRGAP=5, DNAMATRIX:IUB, GAPOPEN=10, GAPEXT=5, MAXDIV=40, TRANSITIONS: unweighted.

Furthermore, identity means that functional and/or structural equivalence exists between the nucleic acid molecules concerned or the proteins coded by them. The nucleic acid molecules, which are homologous to the molecules described above and constitute derivatives of these molecules, are generally variations of these molecules, which constitute modifications, which execute the same biological function. At the same time, the variations can occur naturally, for example they can be sequences from other plant species, or they can be mutants, wherein these mutants may have occurred in a natural manner or have been introduced by objective mutagenesis. The variations can also be synthetically manufactured sequences. The allelic variants can be both naturally occurring variants and also synthetically manufactured variants or variants produced by recombinant DNA techniques. Nucleic acid molecules, which deviate from nucleic acid molecules according to the invention due to degeneration of the genetic code, constitute a special form of derivatives.

The proteins coded from the different derivatives of nucleic acid molecules according to the invention have certain common characteristics. These can include, for example, biological activity, substrate specificity, molecular weight, immunological reactivity, conformation etc, as well as physical characteristics such as, for example, the running behaviour in gel electrophoresis, chromatographic behaviour, sedimentation coefficients, solubility, spectroscopic characteristics, stability; optimum pH, optimum temperature etc. Preferred characteristics of an OK1 protein have already been described in detail above and are to be applied here accordingly.

The nucleic acid molecules according to the invention can be any nucleic acid molecules, in particular DNA or RNA molecules, for example cDNA, genomic DNA,

mRNA etc. They can be naturally occurring molecules or molecules manufactured by genetic engineering or chemical synthesis methods. They can be single-stranded molecules, which either contain the coding or the non-coding strand, or double-stranded molecules.

In a further embodiment, the present invention relates to plant cells according to the invention and plants according to the invention wherein the foreign nucleic acid molecule coding an R1 protein is chosen from the group consisting of

a) Nucleic acid molecules, which code a protein with the amino acid sequence given under SEQ ID NO 7, SEQ ID NO 9, SEQ ID NO 11, SEQ ID NO 13, SEQ ID NO 15 or SEQ ID NO 17,

b) Nucleic acid molecules, which include the nucleotide sequence shown under SEQ ID NO 6, SEQ ID NO 8, SEQ ID NO 10, SEQ ID NO 12, SEQ ID NO 14 or SEQ ID NO 16, or a complimentary sequence;

c) Nucleic acid molecules, the nucleotide sequence of which deviates from the sequence of the nucleic acid molecules identified under a) or b) due to the degeneration of the genetic code;

d) Nucleic acid molecules, which have an identity of at least 70% with the nucleic acid sequences described under a) or b) and

e) Nucleic acid molecules, which hybridise with at least one strand of the nucleic acid molecules described under a) or b) under stringent conditions.

In a further embodiment, the present invention relates to plant cells according to the invention and plants according to the invention wherein the foreign nucleic acid molecule is chosen from the group consisting of

a) T-DNA molecules, which lead to an increase in the expression of an OK1 gene and/or an R1 gene due to integration in the plant genome (T-DNA activation tagging);

b) DNA molecules, which contain transposons, which lead to an increase in the expression of an OK1 gene and/or an R1 gene due to integration in the plant genome (transposon activation tagging);

c) DNA molecules, which code an OK1 protein and/or an R1 protein and which are linked with regulatory sequences, which guarantee transcription in plant cells and lead to an increase in an OK1 protein and/or R1 protein activity in the cell,

d) Nucleic acid molecules introduced by means of *in vivo* mutagenesis, which lead to a mutation or an insertion of a heterologous sequence in at least one endogenous gene coding an OK1 protein, wherein the mutation or insertion effects an increase in the expression of a gene coding an OK1 protein.

e) Nucleic acid molecules introduced by means of *in vivo* mutagenesis, which lead to a mutation or an insertion of a heterologous sequence in at least one endogenous gene coding an R1 protein, wherein the mutation or insertion effects an increase in the expression of a gene coding an R1 protein.

In conjunction with the present invention, plant cells according to the invention and plants according to the invention can also be manufactured by the use of so-called insertion mutagenesis (overview article: Thorneycroft et al., 2001, Journal of experimental Botany 52 (361), 1593-1601). In conjunction with the present invention, insertion mutagenesis is to be understood to mean particularly the insertion of transposons or so-called transfer DNA (T-DNA) into a gene or in the vicinity of a gene coding for an OK1 protein and/or coding for an R1 protein, whereby as a result of which the activity of an OK1 protein and/or an R1 protein in the cell concerned is increased.

The transposons can be both those that occur naturally in the cell (endogenous transposons) and also those that do not occur naturally in said cell but are introduced into the cell (heterologous transposons) by means of genetic engineering methods, such as transformation of the cell, for example. Changing the expression of genes by means of transposons is known to the person skilled in the art. An overview of the

use of endogenous and heterologous transposons as tools in plant biotechnology is presented in Ramachandran and Sundaresan (2001, Plant Physiology and Biochemistry 39, 234-252).

T-DNA insertion mutagenesis is based on the fact that certain sections (T-DNA) of Ti plasmids from Agrobacterium can integrate into the genome of plant cells. The place of integration in the plant chromosome is not defined, but can take place at any point. If the T-DNA integrates into a part of the chromosome or in the vicinity of a part of the chromosome, which constitutes a gene function, then this can lead to an increase in the gene expression and thus also to a change in the activity of a protein coded by the gene concerned.
Here, the sequences inserted into the genome (in particular transposons or T-DNA) are distinguished by the fact that they contain sequences, which lead to an activation of regulatory sequences of an OK1 gene ("activation tagging").

Plant cells and plants according to the invention can be produced by means of the so-called "activation tagging" method (see, for example, Walden et al., Plant J. (1991), 281-288; Walden et al., Plant Mol. Biol. 26 (1994), 1521-1528). These methods are based on activating endogenous promoters by means of enhancer sequences, such as the enhancer of the 35S RNA promoter of the cauliflower mosaic virus, or the octopine synthase enhancer.

In conjunction with the present invention, the term "T-DNA activation tagging" is to be understood to mean a T-DNA fragment, which contains enhancer sequences and which leads to an increase in the activity of at least one OK1 protein and/or at least one R1 protein by integration into the genome of a plant cell.

In conjunction with the present invention, the term "transposon activation tagging" is to be understood to mean a transposon, which contains enhancer sequences and

which leads to an increase in the activity of at least one OK1 protein and/or at least one R1 protein by integration into the genome of a plant cell.

In an additional embodiment, the DNA molecules according to the invention, which code an OK1 protein and/or an R1 protein, are linked with regulatory sequences, which initiate transcription in plant cells and lead to an increase in OK1 protein and/or R1 protein activity in the cell. In this case, the nucleic acid molecules according to the invention are present in "sense" orientation to the regulatory sequences.

For expressing nucleic acid molecules according to the invention, which code an OK1 protein and/or R1 protein, these are preferably linked with regulatory DNA sequences, which guarantee transcription in plant cells. In particular, these include promoters. In general, any promoter that is active in plant cells is eligible for expression.
At the same time, the promoter can be chosen so that expression takes place constitutively or only in a certain tissue, at a certain stage of the plant development or at a time determined by external influences. The promoter can be homologous or heterologous both with respect to the plant and with respect to the nucleic acid molecule.
Suitable promoters are, for example, the promoter of the 35S RNA of the cauliflower mosaic virus and the ubiquitin promoter from maize for constitutive expression, the patatin promoter B33 (Rocha-Sosa et al., EMBO J. 8 (1989), 23-29) for tuber-specific expression in potatoes or a promoter, which only ensures expression in photosynthetically active tissues, e.g. the ST-LS1 promoter (Stockhaus et al., Proc. Natl. Acad. Sci. USA 84 (1987), 7943-7947; Stockhaus et al., EMBO J. 8 (1989), 2445-2451) or, for endosperm-specific expression of the HMG promoter from wheat, the USP promoter, the phaseolin promoter, promoters of zein genes from maize (Pedersen et al., Cell 29 (1982), 1015-1026; Quatroccio et al., Plant Mol. Biol. 15 (1990), 81-93), glutelin promoter (Leisy et al., Plant Mol. Biol. 14 (1990), 41-50; Zheng et al., Plant J. 4 (1993), 357-366; Yoshihara et al., FEBS Lett. 383 (1996), 213-218) or shrunken-1 promoter (Werr et al., EMBO J. 4 (1985), 1373-1380). However,

promoters can also be used, which are only activated at a time determined by external influences (see for example WO 9307279). Promoters of heat-shock proteins, which allow simple induction, can be of particular interest here. Furthermore, seed-specific promoters can be used, such as the USP promoter from *Vicia faba*, which guarantees seed-specific expression in *Vicia faba* and other plants (Fiedler et al., Plant Mol. Biol. 22 (1993), 669-679; Bäumlein et al., Mol. Gen. Genet. 225 (1991), 459-467).

Furthermore, a termination sequence (polyadenylation signal) can be present, which is used for adding a poly-A tail to the transcript. A function in the stabilisation of the transcripts is ascribed to the poly-A tail. Elements of this type are described in the literature (cf. Gielen et al., EMBO J. 8 (1989), 23-29) and can be exchanged at will.

Intron sequences can also be present between the promoter and the coding region. Such intron sequences can lead to stability of expression and to increased expression in plants (Callis et al., 1987, Genes Devel. 1, 1183-1200; Luehrsen, and Walbot, 1991, Mol. Gen. Genet. 225, 81-93; Rethmeier, et al., 1997; Plant Journal. 12(4):895-899; Rose and Beliakoff, 2000, Plant Physiol. 122 (2), 535-542; Vasil et al., 1989, Plant Physiol. 91, 1575-1579; XU et al., 2003, Science in China Series C Vol. 46 No. 6, 561-569). Suitable intron sequences are, for example, the first intron of the sh1 gene from maize, the first intron of the polyubiquitin gene 1 from maize, the first intron of the EPSPS gene from rice or one of the two first introns of the PAT1 gene from Arabidopsis.

Furthermore, plant cells according to the invention and plants according to the invention can be manufactured by means of so-called "*in situ* activation". In this case, the introduced genetic modification effects a change in the regulatory sequences of endogenous OK1 genes and/or R1 genes, which leads to an increased expression of OK1 genes and/or R1 genes. Preferably, the activation of an OK1 gene and/or an R1 gene takes place by "*in vivo*" mutagenesis of a promoter or of enhancer sequences of

an endogenous OK1 gene and/or an R1 gene. In doing so, a promoter or an enhancer sequence, for example, can be changed through mutagenesis in such a way that the mutation produced leads to an increased expression of an OK1 protein gene and/or R1 gene in plant cells according to the invention or plants according to the invention in comparison with the expression of an OK1 gene and/or an R1 gene in wild type plant cells or wild type plants. The mutation in a promoter or an enhancer sequence can also lead to OK1 genes and/or R1 genes in plant cells according to the invention or plants according to the invention being expressed at a time at which they would not be expressed in wild type plant cells or wild type plants.

In conjunction with the present invention, the term "OK1 gene" is to be understood to mean a nucleic acid molecule (cDNA, DNA) that codes an OK1 protein, preferably an OK1 protein from starch-storing plants, particularly preferably from *Arabidopsis thaliana*, especially preferably from rice.

In conjunction with the present invention, the term "R1 gene" is to be understood to mean a nucleic acid molecule (cDNA, DNA) that codes an R1 protein, preferably an R1 protein from starch-storing plants, particularly preferably from *Arabidopsis thaliana*, especially preferably from rice.

In so-called "*in vivo* mutagenesis", a hybrid RNA-DNA oligonucleotide ("Chimeroplast") is introduced into plant cells through transformation of plants cells (Kipp, P.B. et al., Poster Session at the "5th International Congress of Plant Molecular Biology", 21st-27th September 1997, Singapore; R. A. Dixon and C.J. Arntzen, meeting report on "Metabolic Engineering in Transgenic Plants", Keystone Symposia, Copper Mountain, CO, USA, TIBTECH 15, (1997), 441-447; international patent application WO 9515972; Kren et al., Hepatology 25, (1997), 1462-1468; Cole-Strauss et al., Science 273, (1996), 1386-1389; Beetham et al., 1999, PNAS 96, 8774-8778).

A part of the DNA components of the RNA-DNA oligonucleotide is homologous to a nucleic acid sequence of an endogenous OK1 gene and/or R1 gene, but, in comparison with the nucleic acid sequence of an endogenous OK1 gene and/or R1 gene, it has a mutation or contains a heterologous region, which is surrounded by the homologous regions.

By base pairing of the homologous regions of the RNA-DNA oligonucleotide and the endogenous nucleic acid molecule followed by homologous recombination, the mutation or heterologous region contained in the DNA components of the RNA-DNA oligonucleotide can be transferred into the genome of a plant cell. This leads to an increase of the activity of one or more OK1 proteins.

All these methods are based on the introduction of a foreign nucleic acid molecule into the genome of a plant cell or plant and are therefore basically suitable for the manufacture of plant cells according to the invention and plants according to the invention.

Surprisingly, it has been found that plant cells according to the invention and plants according to the invention synthesise a modified starch in comparison with starch of corresponding wild type plant cells or wild type plants that have not been genetically modified.

The plant cells according to the invention and plants according to the invention synthesise a modified starch, which in its physical-chemical characteristics, in particular the starch phosphate content and/or phosphate distribution is changed in comparison with the starch synthesised in wild type plant cells or wild type plants, so that this is better suited for special applications.

Because no enzymes have yet been described that exclusively phosphorylate P-starch, it has also previously not been possible to increase beyond a certain quantity the concentration of starch phosphate of starch that is already phosphorylated in

plants. This is only possible by using an enzyme with the function of an OK1 protein or by preparing a nucleic acid molecule that codes an OK1 protein for the genetic modification of plants.

The phosphate distribution of starch synthesised by plants was also not previously possible due to the lack of available means. A change of the phosphate ratio of native starches is also now possible through the present invention through the preparation of enzymes with the function of OK1 proteins and the preparation of nucleic acid molecules that code an OK1 protein.

The present invention therefore also includes plant cells according to the invention and plants according to the invention, which synthesise a modified starch, in comparison with corresponding wild type plant cells or wild type plans that have not been genetically modified.

In conjunction with the present invention, the term "modified starch" means that the starch has changed physical-chemical characteristics compared with non-modified starch obtainable from corresponding wild type plant cells or wild type plants.

In a further embodiment of the present invention, plant cells according to the invention or plants according to the invention synthesise a starch, which has an increased concentration of starch phosphate and/or a changed phosphate distribution in comparison with starch isolated from corresponding wild type plant cells or wild type plants.

In conjunction with the present invention, the term "phosphate distribution" is to be understood to mean the proportion of the starch phosphate bound to a glucose molecule in the C-2 position, C-3 position or C-6 position in terms of the total concentration of starch phosphate from starch.

In a further embodiment of the present invention, plant cells according to the invention or plants according to the invention synthesise a starch, which has an increased concentration of starch phosphate and/or a changed ratio of C-3 phosphate to C-6 phosphate in comparison with starch from wild type plants that have not been genetically modified. Preferred here are starches, which have an increased proportion of starch phosphate bonded in the C-3 position compared with starch phosphate bonded in the C-6 position in comparison with starches from wild type plant cells that have not been genetically modified or wild type plants that have not been genetically modified.

In conjunction with the present invention, the term "ratio of C-3 phosphate to C-6 phosphate" is to be understood to mean the proportion of starch phosphate for which the starch phosphate of a starch bonded in the C-3 position or C-6 position respectively adds to the total of the starch phosphate of the starch concerned that is bonded in the C-3 position and in the C-6 position (C-3 position + C-6 position).

Various methods are described for the determination of the amount of starch phosphate. Preferably, the methods described by Ritte et al. (2000, Starch/Stärke 52, 179-185) can be used for the determination of the amount of starch phosphate. Particularly preferably, the determination of the amount of starch phosphate is carried out by means of ^{31}P-NMR according to the methods described by Kasemusuwan and Jane (1996, Cereal Chemistry 73, 702-707).

Furthermore, genetically modified plants, which contain the plant cells according to the invention, are also the subject matter of the invention. Plants of this type can be produced from plant cells according to the invention by regeneration.

In principle, the plants according to the invention can be plants of any plant species, i.e. both monocotyledonous and dicotyledonous plants. Preferably they are useful

plants, i.e. plants, which are cultivated by people for the purposes of food or for technical, in particular industrial, purposes.

In a further embodiment, the plant according to the invention is a starch-storing plant.

In conjunction with the present invention, the term "starch-storing plants" means all plants with plant parts, which contain a storage starch, such as, for example, maize, rice, wheat, rye, oats, barley, cassava, potato, sago, mung bean, pea or sorghum.

In conjunction with the present invention, the term "potato plant" or "potato" means plant species of the genus *Solanum*, in particular tuber-producing species of the genus *Solanum* and especially *Solanum tuberosum*.

In conjunction with the present invention, the term "wheat plant" means plant species of the genus *Triticum* or plants resulting from crosses with plants of the genus *Triticum*, particularly plant species of the genus *Triticum* or plants resulting from crosses with plants of the genus *Triticum*, which are used in agriculture for commercial purposes, and particularly preferably *Triticum aestivum*.

In conjunction with the present invention, the term "maize plant" means plant species of the genus *Zea*, particularly plant species of the genus *Zea*, which are used in agriculture for commercial purposes, particularly preferably *Zea mais*.

In a further embodiment, the present invention relates to starch-storing plants of the (systematic) family *Poaceae* according to the invention. Preferably these are here maize- or wheat plants.

The present invention also relates to propagation material of plants according to the invention containing a plant cell according to the invention.

Here, the term "propagation material" includes those constituents of the plant that are suitable for producing offspring by vegetative or sexual means. Cuttings, callus cultures, rhizomes or tubers, for example, are suitable for plant propagation. Other propagation material includes, for example, fruits, seeds, seedlings, protoplasts, cell cultures, etc. Preferably, the propagation material is tubers and particularly preferably grains, which contain endosperms.

In a further embodiment, the present invention relates to harvestable plant parts of plants according to the invention such as fruits, storage roots, roots, blooms, buds, shoots or stems, preferably seeds, grains or tubers, wherein these harvestable parts contain plant cells according to the invention.

Furthermore, the present invention also relates to a method for the manufacture of a genetically modified plant according to the invention, wherein

a) a plant cell is genetically modified, wherein the genetic modification leads to an increase in the enzymatic activity of an OK1 protein and an R1 protein in comparison with corresponding wild type plant cells that have not been genetically modified;
b) a plant is regenerated from plant cells from step a);
c) and, if necessary, further plants are produced with the help of the plants according to Step b).

The genetic modification introduced into the plant cell according to Step a) can basically be any type of genetic modification, which leads to the increase of the activity of an OK1 protein and an R1 protein. The regeneration of the plants according to Step (b) can be carried out using methods known to the person skilled in the art (e.g. described in "Plant Cell Culture Protocols", 1999, edt. by R.D. Hall, Humana Press, ISBN 0-89603-549-2).

The production of further plants according to Step (c) of the method according to the invention can be carried out, for example, by vegetative propagation (for example using cuttings, tubers or by means of callus culture and regeneration of whole plants) or by sexual propagation. Here, sexual propagation preferably takes place under controlled conditions, i.e. selected plants with particular characteristics are crossed and propagated with one another. In this case, the selection is preferably carried out in such a way that further plants, which are produced in accordance with Step c), exhibit the modification, which was introduced in Step a).

The genetic modifications for the production of the plant cells according to the invention can take place simultaneously or in successive steps. At the same time, the genetic modification can be any genetic modification, which leads to the increase of the activity of at least one OK1 protein and/or at least one R1 protein. It can come from wild type plants as well as wild type plant cells, in which no prior genetic modification for the reduction of the activity of at least one OK1 protein or at least one R1 protein has yet taken place, or from plant cells or plants that are already genetically modified, in which the activity of at least one OK1 protein or at least one R1 protein is already increased through a genetic modification. It is irrelevant whether the same method is used for the genetic modification that leads to an increased activity of an OK1 protein as for the genetic modification that leads to an increased activity of an R1 protein, so long as both genetic modifications together lead to an increased activity of an OK1 protein and an R1 protein in the same plant cell.

In a further embodiment of the method according to the invention for the manufacture of a genetically modified plant according to the invention, the genetic modification consists in the introduction of at least one foreign nucleic acid molecule into the genome of the plant cell, wherein the availability or the expression of foreign nucleic acid molecule(s) lead(s) to an increased activity of an OK1 protein and an R1 protein in the cell.

In a further embodiment of the method according to the invention for the manufacture of a genetically modified plant according to the invention, the genetic modification consists in the introduction of at least one foreign nucleic acid molecule into the genome of the plant cell, wherein the foreign nucleic acid molecule(s) contain(s) a sequence coding an OK1 protein and/or an R1 protein.

As already described above for foreign nucleic acid molecules assembled for the genetic modification in the plant cell or plant, Step a) of the method according to the invention for the manufacture of a genetically modified plant according to the invention can involve a single nucleic acid molecule or multiple nucleic acid molecules. The embodiments provided above are to be correspondingly applied for the method according to the invention described here.

In a further embodiment of the method according to the invention for the manufacture of a genetically modified plant according to the invention, the genetic modification in Step a) of the method consists in the introduction of a foreign nucleic acid molecule which contains at least one sequence coding R1 protein and at least one sequence coding OK1 protein.

In a further embodiment of the method according to the invention for the manufacture of a genetically modified plant according to the invention, the genetic modification in Step a) of the method consists in the introduction of multiple foreign nucleic acid molecules, wherein at least one first nucleic acid molecule contains a sequence coding an R1 protein and at least a second nucleic acid molecule contains a sequence coding an OK1 protein.

Furthermore, instead of a wild type plant cell or wild type plant, a mutant cell or a mutant characterised in that it already has an increased activity of an OK1 protein or an increased activity of an R1 protein can be used for introducing a foreign nucleic acid molecule for the implementation of the method according to the invention. The

additional information provided above for the use of mutants for the manufacture of plant cells according to the invention or plant are to be correspondingly applied here.

In a further embodiment of the method according to the invention for the manufacture of a genetically modified plant according to the invention, at least one foreign nucleic acid molecule is selected from the group consisting of

a) Nucleic acid molecules, which code a protein with the amino acid sequence given under SEQ ID NO 2 or SEQ ID NO 4;

b) Nucleic acid molecules, which code a protein, which includes the amino acid sequence, which is coded by the insertion in plasmid A.t.-OK1-pGEM or the insertion in plasmid pMI50;

c) Nucleic acid molecules, which code a protein, the sequence of which has an identity of at least 60% with the amino acid sequence given under SEQ ID NO 2 or SEQ ID NO 4;

d) Nucleic acid molecules, which code a protein, the sequence of which has an identity of at least 60% with the amino acid sequence, which is coded by the insertion in plasmid A.t.-OK1-pGEM or by the insertion in plasmid pMI50;

e) Nucleic acid molecules, which include the nucleotide sequence shown under SEQ ID NO 1 or SEQ ID NO 3 or a complimentary sequence;

f) Nucleic acid molecules, which include the nucleotide sequence of the insertion contained in plasmid A.t.-OK1-pGEM or plasmid pMI50;

g) Nucleic acid molecules, which have an identity of at least 70% with the nucleic acid sequences described under a), b), e) or f);

h) Nucleic acid molecules, which hybridise with at least one strand of the nucleic acid molecules described under a), b), e) or f) under stringent conditions;

i) Nucleic acid molecules, the nucleotide sequence of which deviates from the sequence of the nucleic acid molecules identified under a), b), e) or f) due to the degeneration of the genetic code; and

j) Nucleic acid molecules, which represent fragments, allelic variants and/or derivatives of the nucleic acid molecules identified under a), b), c), d), e), f), g), h) or i).

In a further embodiment of the method according to the invention for the manufacture of a genetically modified plant according to the invention, an R1 protein from potato, wheat, rice, maize, soybean, citrus or *Arabidopsis* codes at least one foreign nucleic acid molecule. References for the identified nucleic acid sequences coding R1 proteins from the identified plants have already been further specified above.

A further embodiment of the present invention also relates to a method for the manufacture of a genetically modified plant according to the invention, wherein

a) a plant cell is genetically modified, wherein the genetic modification leads to an increase in the enzymatic activity of an OK1 protein in comparison with corresponding wild type plant cells that have not been genetically modified;
b) a plant is regenerated from plant cells from Step a);
c) if necessary, further plants are produced with the help of the plants according to Step b) and
d) Plants obtained according to Step b) or c) are crossed with a plant, which has an increased level of enzymatic activity of an R1 protein, in comparison with corresponding wild type plant cells that have not been genetically modified.

A further embodiment of the present invention relates to a method for the manufacture of a genetically modified plant according to the invention, wherein

a) a plant cell is genetically modified, wherein the genetic modification leads to an increase in the enzymatic activity of an R1 protein in comparison with corresponding wild type plant cells that have not been genetically modified;
b) a plant is regenerated from plant cells from Step a);
c) if necessary, further plants are produced with the help of the plants according to Step b) and

d) Plants obtained according to Step b) or c) are crossed with a plant, which has an increased level of enzymatic activity of an OK1 protein, in comparison with corresponding wild type plant cells that have not been genetically modified.

At the same time, the plants according to Step a) can be genetically modified as already described above. The regeneration of plans according to Step b) and the production of additional plants according to Step c) was also already further presented above.

A plant that is crossed with plants or offspring of plants obtained in Step b) or c) according to Step d) of the two most recently identified embodiments, can be any plant that has an increased activity of an OK1 protein or an R1 protein, in comparison to corresponding wild type plants. The increase of the activity of an OK1 protein or an R1 protein can be brought about through any modification that leads to an increase of the activity of the related protein in the corresponding plants. These plants can be mutants or plants modified by means of genetic engineering methods. Mutants can be spontaneously (naturally) occurring mutants as well as those that were produced through the targeted use of mutagens (such as, for example, chemical agents, ionising radiation) or genetic engineering methods (for example transposon activation tagging, T-DNA activation tagging, *in vivo* mutagenesis). Preferably, the plants produced through genetic engineering methods are mutants manufactured by means of insertion mutagenesis, particularly preferably genetically modified plants that express a foreign nucleic acid molecule, especially preferably genetically modified plants in which the foreign nucleic acid molecule codes an OK1 protein or an R1 protein.

In a further embodiment, the method according to the invention is used for manufacturing a genetically modified plant according to the invention for producing starch-storing plants.

In a further embodiment, the method according to the invention is used for manufacturing a genetically modified plant according to the invention for producing maize- or wheat plants according to the invention.

In a further embodiment, the present invention relates to a method according to the invention for manufacturing a genetically modified plant according to the invention, wherein the genetically modified plant synthesises a modified starch in comparison with wild type plants that have not been genetically modified.

In a further embodiment of the method according to the invention for manufacturing a genetically modified plant, the plants according to the invention synthesise a modified starch, which has an increased concentration of starch phosphate and/or a changed starch phosphate distribution in comparison with starch isolated from corresponding wild type plants.

In a further embodiment of the method according to the invention for manufacturing a genetically modified plant, the plants according to the invention synthesise a modified starch, which has a changed ratio of C-3 phosphate to C-6 phosphate in comparison with starch from wild type plants that have not been genetically modified. Especially preferred here are starches, which have an increased proportion of starch phosphate bonded in the C-3 position compared with starch phosphate bonded in the C-6 position in comparison with starches from wild type plant that have not been genetically modified.

The present invention also relates to plants ohbtainable by the methods according to the invention.

Surprisingly, it has been found that starch isolated from plant cells according to the invention and plants according to the invention, which have an increased activity of an OK1 protein and an increased activity of an R1 protein, synthesise a modified starch..

In particular, the increased amount of starch phosphate in starches according to the invention gives the starches surprising and advantageous characteristics. By means of the increased proportion of starch phosphate, starches according to the invention support an increased proportion of charged groups that significantly affect the functional characteristics of the starch. Starch that supports the charged functional groups is particularly applicable in the paper industry, where it is used for the coating of paper. Paper that otherwise has good adhesive characteristics with charged molecules is particularly suitable for the absorption of dyestuffs, such as ink, print colors, etc., when coated.

The present invention also relates to modified starches obtainable from plant cells according to the invention or plants according to the invention, from propagation material according to the invention or from harvestable plant parts according to the invention.

In a further embodiment, the present invention relates to modified starch according to the invention from starch-storing plants, preferably from starch-storing plants of the (systematic) family *Poaceae*, particularly preferably from maize or wheat plants.

Furthermore, the present invention relates to a method for the manufacture of a modified starch including the step of extracting the starch from a plant cell according to the invention or from a plant according to the invention, from propagation material according to the invention of such a plant and/or from harvestable plant parts according to the invention of such a plant, preferably from starch-storing parts according to the invention of such a plant. Preferably, such a method also includes the step of harvesting the cultivated plants or plant parts and/or the propagation material of these plants before the extraction of the starch and, further, particularly preferably the step of cultivating plants according to the invention before harvesting.

Methods for extracting starches from plants or from starch-storing parts of plants are known to the person skilled in the art. Furthermore, methods for extracting starch from different starch-storing plants are described, e.g. in Starch: Chemistry and Technology (Publisher: Whistler, BeMiller and Paschall (1994), 2nd Edition, Academic Press Inc. London Ltd; ISBN 0-12-746270-8; see e.g. Chapter XII, Page 412-468: Maize and Sorghum Starches: Manufacture; by Watson; Chapter XIII, Page 469-479: Tapioca, Arrowroot and Sago Starches: Manufacture; by Corbishley and Miller; Chapter XIV, Page 479-490: Potato starch: Manufacture and Uses; by Mitch; Chapter XV, Page 491 to 506: Wheat starch: Manufacture, Modification and Uses; by Knight and Oson; and Chapter XVI, Page 507 to 528: Rice starch: Manufacture and Uses; by Rohmer and Klem; Maize starch: Eckhoff et al., Cereal Chem. 73 (1996), 54-57, the extraction of maize starch on an industrial scale is generally achieved by so-called "wet milling".). Devices, which are in common use in methods for extracting starch from plant material are separators, decanters, hydrocyclones, spray dryers and fluid bed dryers.

In conjunction with the present invention, the term "starch-storing parts" is to be understood to mean such parts of a plant in which, in contrast to transitory leaf starch, starch is stored as a deposit for surviving for longer periods. Preferred starch-storing plant parts are, for example, tubers, storage roots and grains, particularly preferred are grains containing an endosperm, especially particularly preferred are grains containing an endosperm of maize or wheat plants.

Modified starch obtainable by a method according to the invention for manufacturing modified starch is also the subject matter of the present invention.

In a further embodiment of the present invention, the modified starch according to the invention is native starch.

In conjunction with the present invention, the term "native starch" means that the starch is isolated from plants according to the invention, harvestable plant plants according to the invention, starch-storing parts according to the invention or propagation material of plants according to the invention by methods known to the person skilled in the art.

Furthermore, the use of plant cells according to the invention or plants according to the invention for manufacturing a modified starch are the subject matter of the present invention.

The person skilled in the art knows that the characteristics of starch can be changed by thermal, chemical, enzymatic or mechanical derivation, for example. Derived starches are particularly suitable for different applications in the foodstuffs and/or non-foodstuffs sector. The starches according to the invention are better suited as a starting substance for the manufacture of derived starches than conventional starches, as they have a higher proportion of reactive functional groups due to the higher starch phosphate content.

The present invention therefore also relates to the manufacture of a derived starch, wherein modified starch according to the invention is derived retrospectively.

In conjunction with the present invention, the term "derived starch" is to be understood to mean a modified starch according to the invention, the characteristics of which have been changed after isolation from plant cells with the help of chemical, enzymatic, thermal or mechanical methods.

In a further embodiment of the present invention, the derived starch according to the invention is starch that has been treated with heat and/or acid.

In a further embodiment, the derived starches are starch ethers, in particular starch alkyl ethers, O-allyl ethers, hydroxylalkyl ethers, O-carboxylmethyl ethers, nitrogen-containing starch ethers, phosphate-containing starch ethers or sulphur-containing starch ethers.

In a further embodiment, the derived starches are crosslinked starches.

In a further embodiment, the derived starches are starch graft polymers.

In a further embodiment, the derived starches are oxidised starches.

In a further embodiment, the derived starches are starch esters, in particular starch esters, which have been introduced into the starch using organic acids. Particularly preferably these are phosphate, nitrate, sulphate, xanthate, acetate or citrate starches.

The derived starches according to the invention are suitable for different applications in the pharmaceutical industry and in the foodstuffs and/or non-foodstuffs sector. Methods for manufacturing derived starches according to the invention are known to the person skilled in the art and are adequately described in the general literature. An overview on the manufacture of derived starches can be found, for example, in Orthoefer (in Corn, Chemistry and Technology, 1987, eds. Watson und Ramstad, Chapter 16, 479-499).

Derived starch obtainable by the method according to the invention for manufacturing a derived starch is also the subject matter of the present invention.

Furthermore, the use of modified starches according to the invention for manufacturing derived starch is the subject matter of the present invention.

Starch-storing parts of plants are often processed into flours. Examples of parts of plants from which flours are manufactured are, for example, tubers of potato plants and grains from cereal plants. The grains of these plants that contain endosperms are milled and sifted for the manufacture of flours from cereal plants. Starch is a principal component of endosperm. For other plants that contain no endosperm, but rather other starch-storing parts, such as tubers or roots, flour is commonly manufactured by reducing, drying and then milling the storage organs concerned. The starch of the endosperm or contained in starch-storing parts of plants is an important part of flour, which is manufactured from the plant parts concerned. The characteristics of flours are therefore also affected by the starch present in the flour concerned. Plant cells according to the invention and plants according to the invention synthesise a modified starch in comparison with corresponding wild type plant cells that have not been genetically modified or wild type plants that have not been genetically modified. Flours manufactured from plant cells according to the invention, plants according to the invention, propagation material according to the invention or harvestable parts according to the invention therefore have modified characteristics. The characteristics of flours can also be affected by mixing starch with flours or by mixing flours with different characteristics.

A further subject of the present invention therefore relates to flours containing a starch according to the invention.

A further subject of the present invention relates to flours that are manufactured from plant cells according to the invention, plants according to the invention, starch-storing parts of plants according to the invention, from propagation material according to the invention or from harvestable plant parts according to the invention. Preferred starch-storing parts of plants according to the invention are tubers, storage roots and a grain, which contains endosperm. Tubers from potato plants and grains preferably originate from plants of the (systematic) family *Poaceae*; grains particularly preferably originate from maize or wheat plants.

In conjunction with the present invention, the term "flour" means a powder obtained by milling plant parts. If necessary, plant parts are dried before milling and reduced and/or sifted after milling.

Flours according to the invention are particularly distinguished by their increased water binding capacity due to the starch present in them that has a modified phosphate content and/or a modified phosphate distribution. This is, for example, desired for the processing of flours in the food industry for many applications, in particular in the manufacture of bakery products.

A further subject of the present invention is a method for the manufacture of flours, including the step of the milling of plant cells according to the invention, plants according to the invention, of parts of plants according to the invention, starch-storing parts of plants according to the invention, propagation material according to the invention or harvestable material according to the invention.

Flours can be manufactured through the milling of starch-storing parts of according to the invention. It is known to the person skilled in the art how to manufacture flours. Preferably, a method for the manufacture of flours also includes the step of harvesting the cultivated plants or plant parts and/or of the propagation material or the starch-storing parts of these plants before the milling and particularly preferably further the step of cultivating plants according to the invention before harvesting.

In conjunction with the present invention the term "parts of plants" is to be understood to mean all parts of a plant, which represent a complete plant as constituents in their totality. Parts of plants are, for example, shoots, leaves, rhizomes, roots, beetroots, tubers, pods, seeds or grains.

A further subject of the present invention includes the method for the manufacture of flours, of a processing of plants according to the invention, of starch-storing parts of plants according to the invention, of propagation material according to the invention or of material according to the invention harvestable before milling.

At the same time, the processing can be, for example, a heating treatment and/or a drying. Heating treatment followed by a drying of the heat-treated material is used, for example, for the manufacture of flours from storage roots or tubers such as, for example, from potato tubers, before which the milling takes place. The reduction of plants according to the invention, of starch-storing parts of plants according to the invention, of propagation material according to the invention or of material according to the invention harvestable before milling can likewise represent a processing in terms of the present invention. The removal of plant tissue such as, for example, of husks of grains, before the milling also represents a processing before the milling in terms of the present invention.

A further embodiment of the present invention includes the method for the manufacture of flours after the milling of a product of the grist processing.
At the same time, the grist can be, for example, sifted after the milling in order to manufacture, for example, various types of flours.

A further subject of the present invention is the use of genetically modified plant cells according to the invention, plants according to the invention, of parts of plants according to the invention, starch-storing parts of plants according to the invention, propagation material according to the invention or harvestable material according to the invention for the manufacture of flours.

It is also an object of the present invention to provide means such as DNA molecules, for example, for the production of plant cells according to the invention and plants according to the invention, which synthesise a modified starch in comparison with modified wild type plant cells or wild type plants that have not been genetically

modified. The provided DNA molecules contain nucleic acid sequences which code an OK1 protein. A protein with the enzymatic activity of an OK1 protein was not previously known to the person skilled in the art. Also, no DNA molecules can thus be provided, which allow plant cells according to the invention and plants according to the invention and the starch synthesised from them and the flours extracted from them to be produced.

Consequently, the present invention also relates to a recombinant nucleic acid molecule containing a nucleic acid sequence coding an OK1 protein and a nucleic acid sequence coding an R1 protein.

In conjunction with the present invention, the term "recombinant nucleic acid molecule" is to be understood to mean a nucleic acid molecule that contains both nucleic acid sequences coding an OK1 protein as well as nucleic acid sequences coding an R1 protein and in which the nucleic acid sequences coding an OK1 protein and an R1 protein are present in an arrangement as they are not present naturally in the genome of an organism. In addition to nucleic acid sequences coding an OK1 protein and nucleic acid sequences coding an R1 protein, the recombinant nucleic acid molecule can still contain additional sequences, which are not naturally present in one such arrangement as they are present in recombinant nucleic acid molecules according to the invention. At the same time, the said additional sequences can be any sequences, preferably regulatory sequences (promoters, termination signals, enhancers), particularly preferably regulatory sequences, which are active in plant tissue, particularly preferably regulatory sequences, which are active in starch-storing plant tissue. Methods for producing recombinant nucleic acid molecules according to the invention are known to the person skilled in the art and include genetic engineering methods such as, for example, the linking of nucleic acid molecules by ligation, genetic recombination or the resynthesis of nucleic acid molecules (see, for example, Sambrok et al., Molecular Cloning, A Laboratory Manual, 3rd edition (2001) Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY. ISBN: 0879695773,

Ausubel et al., Short Protocols in Molecular Biology, John Wiley & Sons; 5th edition (2002), ISBN: 0471250929).

A further embodiment of the present invention of recombinant nucleic acid molecules according to the invention comprises vectors, in particular plasmids, cosmids, viruses, bacteriophages and other common vectors in genetic engineering, which contain the nucleic acid molecules according to the invention described above.

In a further embodiment, the nucleic acid molecules according to the invention contained in the vectors are linked with regulatory sequences, which initiate the expression in prokaryotic or eukaryotic cells. At the same time, the term "expression" can mean transcription as well as transcription and translation. In this case, the nucleic acid molecules according to the invention can be present in "sense" orientation and/or in "antisense" orientation to the regulatory sequences. At the same time, the recombinant nucleic acid molecules according to the invention can collectively remain under the control of a single regulatory element, or they can each have their own respective individual regulatory element.

Regulatory sequences for expression in prokaryotic organisms, e.g. *E. coli*, and in eukaryotic organisms are adequately described in the literature, in particular those for expression in yeast such as *Saccharomyces cerevisiae*, for example. An overview of different expression systems for proteins and different host organisms can be found, for example, in Methods in Enzymology 153 (1987), 383-516 and in Bitter et al. (Methods in Enzymology 153 (1987), 516-544).

A further subject of the present invention is a host cell, in particular a prokaryotic or eukaryotic cell, which is genetically modified with a recombinant nucleic acid molecule according to the invention and/or with a vector according to the invention, as well as cells, which originate from host cells of this type and which contain the genetic modification according to the invention.

In a further embodiment, the invention relates to host cells, particularly prokaryotic or eukaryotic cells, which were transformed with a nucleic acid molecule according to the invention or a vector according to the invention, as well as host cells, which originate from host cells of this type and which contain the described nucleic acid molecules according to the invention or vectors.

The host cells can be bacteria (e.g. *E. coli*, bacteria of the genus *Agrobacterium* in particular *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*) or fungus cells (e.g. yeast, in particular *S. cerevisiae*, *Agaricus*, in particular *Agaricus bisporus, Aspergillus, Trichoderma*), as well as plant or animal cells. Here, the term "transformed" means that the cells according to the invention are genetically modified with a nucleic acid molecule according to the invention inasmuch as they contain at least one nucleic acid molecule according to the invention in addition to their natural genome. This can be freely present in the cell, possibly as a self-replicating molecule, or it can be stably integrated in the genome of the host cell.
The host cells are preferably microorganisms. Within the framework of the present application, these are understood to mean all bacteria and all protista (e.g. fungi, in particular yeast and algae), as defined, for example, in Schlegel "Allgemeine Mikrobiologie" (Georg Thieme Verlag (1985), 1-2).

It is preferred if the host cells according to the invention are plant cells. In principle, these can be plant cells from any plant species, i.e. both monocotyledonous and dicotyledonous plants. Preferably, these will be plant cells from useful agricultural plants, i.e. from plants, which are cultivated by people for the purposes of food or for technical, in particular industrial purposes. The invention relates preferably to plant cells and plants from starch-storing plants (maize, rice, wheat, rye, oat, barley, cassava, potato, sago, mung bean, pea or sorghum), preferably plant cells from plants of the (systematic) family *Poacea*, especially particularly preferred are plant cells from maize or wheat plants.

Also the subjects of the present invention are compositions containing a recombinant nucleic acid molecule according to the invention, or a vector according to the invention. Preferred are compositions according to the invention containing a recombinant nucleic acid molecule according to the invention, or a vector according to the invention and a host cell. It is particularly preferred if the host cell is a plant cell, and especially preferred if it is a cell of a maize or wheat plant.

A further subject of the present invention relates to a composition containing a nucleic acid sequence coding an OK1 protein and a nucleic acid sequence coding an R1 protein.
At the same time, the nucleic acid sequences coding an OK1 protein or coding an R1 protein can exist together in a single nucleic acid molecule, or in nucleic acid molecules separated from one another.

A further aspect of compositions according to the invention relates to compositions, which can be used for producing host cells according to the invention, preferably for producing plant cells according to the invention. Preferably this concerns a composition containing nucleic acid sequences coding an OK1 protein and nucleic acid sequences coding an R1 protein, a recombinant nucleic acid molecule according to the invention or a vector according to the invention and a biolistic carrier, which is suitable for the introduction of nucleic acid molecules into a host cell. Preferred biolistic carriers are particles of tungsten, gold or synthetic materials.

A further embodiment of compositions according to the invention relates to compositions containing nucleic acid sequences coding an OK1 protein and nucleic acid sequences coding an R1 protein, a recombinant nucleic acid molecule according to the invention or a vector according to the invention and a plant cell and a synthetic cultivation medium. Preferably such compositions also contain polyethylene glycol (PEG) in addition to plant cells and synthetic cultivation medium. With these compositions, the recombinant nucleic acid molecule exists outside the plant cell, i.e.

it is situated outside the cell interior of the plant cell, which is enclosed by a cytoplasmic membrane.

Synthetic cultivation media, which are suitable for the cultivation and/or transformation of plant cells, are known to the person skilled in the art and are adequately described in the literature, for example. Many different synthetic cultivation media are also available for purchase in the specialised trade (e.g. DUCHEFA Biochemie B.V., Belgium).

Furthermore, the present invention relates to the use of compositions according to the invention for the transformation of plant cells.

Description of sequences

SEQ ID NO 1: Nucleic acid sequence containing the coding region of an A.t.-OK1 protein from *Arabidopsis thaliana*. This sequence is inserted in the vectors OK1-pGEM-T and OK1-pDEST™17.

SEQ ID NO 2: Amino acid sequence coding an A.t.-OK1 protein from *Arabidopsis thaliana*. This sequence can be derived from the nucleic acid sequence shown under SEQ ID NO 1.

SEQ ID NO 3: Nucleic acid sequence containing the coding region of an O.s.-OK1 protein from *Oryza sativa*. The sequence is inserted in vector pMI50.

SEQ ID NO 4: Amino acid sequence coding an O.s.-OK1 protein from *Oryza sativa*. This sequence can be derived from the nucleic acid sequence shown under SEQ ID NO 3.

SEQ ID NO 5: Peptide sequence coding the phosphohistidine domain of the OK1 proteins from *Arabidopsis thaliana* and *Oryza sativa*.

SEQ ID NO 6: Nucleic acid sequence containing the coding region of a C.r.-R1 protein from *Citrus reticulata*.

SEQ ID NO 7: Amino acid sequence coding a C.r.-R1 protein from *Citrus reticulata*.

SEQ ID NO 8: Nucleic acid sequence containing the coding region of an A.t.-R1 protein from *Arabidopsis thaliana*.

SEQ ID NO 9: Amino acid sequence coding an A.t.-R1 protein from *Arabidopsis thaliana*.

SEQ ID NO 10: Nucleic acid sequence containing the coding region of an S.t.-R1 protein from *Solanum tuberosum*.

SEQ ID NO 11: Amino acid sequence coding an S.t.-R1 protein from *Solanum tuberosum*.

SEQ ID NO 12: Nucleic acid sequence containing the coding region of an O.s.-R1 protein from *Oryza sativa*.

SEQ ID NO 13: Amino acid sequence coding an O.s.-R1 protein from *Oryza sativa*.

SEQ ID NO 14: Nucleic acid sequence containing the coding region of a G.m.-R1 protein from *Glycine max*.

SEQ ID NO 15: Amino acid sequence coding the S.t.-R1 protein from *Glycine max*.

SEQ ID NO 16: Nucleic acid sequence containing a coding region of a Z.m.-R1 protein from *Zea mays*.

SEQ ID NO 17: Amino acid sequence coding a Z.m-R1 protein from *Zea mays*.

Description of Figures

Fig. 1: Denaturing acrylamide gel for identifying proteins from *Arabidopsis thaliana*, which preferably bond to non-phosphorylated starch in comparison with phosphorylated starch. Standard protein molecular weight marker is shown in trace "M". Proteins obtained after incubation of control preparation C from Example 1 d) are shown in trace "-". Protein extracts of *Arabidopsis thaliana*, obtained after incubation with non-phosphorylated starch, isolated from leaves of an *Arabidopsis thaliana sex1-3* mutant (Preparation B, Example 1 d), are shown in trace "K". Protein extracts of *Arabidopsis thaliana*, obtained after incubation with non-phosphorylated starch, isolated from leaves of an *Arabidopsis thaliana sex1-3* mutant, which was phosphorylated retrospectively *in vitro* with an R1 protein (Preparation A, Example 1 d), are shown in trace "P". On completion of electrophoresis, the acrylamide gel was stained with Coomassie Blue.

Fig. 2: Demonstration of the autophosphorylation of the OK1 protein. Fig. 2 A) shows a denaturing (SDS) acrylamide gel on completion of electrophoresis stained with Coomassie Blue. Fig. 2 B) shows the autoradiography of a denaturing (SDS) acrylamide gel. The same amounts of the same samples were applied to each of the two gels. M: Standard protein molecular weight marker; R1: Sample from reaction vessel 1 according to Example 7 (after incubating an

OK1 protein with ATP); R2: Sample from reaction vessel 2 according to Example 7 (after incubating an OK1 protein with ATP the protein was heated to 95°C); R3: Sample from reaction vessel 3 according to Example 7 (after incubating an OK1 protein with ATP the protein was incubated in 0.5 M HCl); R4: Sample from reaction vessel 4 according to Example 7 (after incubating an OK1 protein with ATP the protein was incubated in 0.5 M NaOH).

Fig. 3: Demonstration of the starch-phosphorylating activity of an OK1 protein (see Example 6). OK1 protein was incubated with non-phosphorylating starch isolated from leaves of an *Arabidopsis thaliana sex1-3* mutant (Preparation A) and starch isolated from leaves of an *Arabidopsis thaliana sex1-3* mutant, which was phosphorylated retrospectively *in vitro* with an R1 protein (Preparation B). Preparation C is the same as Preparation B, except that this Preparation C was incubated without OK1 protein. Two independent tests were carried out for each preparation (A, B, C) (Test 1 and Test 2). The respective amounts are shown graphically, measured in cpm (counts per minute), on ^{33}P labeled phosphate, which was introduced into non-phosphorylated starch (Preparation A) and phosphorylated starch (Preparation B) from the OK1 protein.

Fig. 4: Comparison of the C-atom positions of glucose molecules of the starch, which was phosphorylated from an R1 protein and an OK1 protein respectively (see Example 9). OK1 protein (Preparation A) was incubated in the presence of ATP labeled with ^{33}P with starch isolated from leaves of an *Arabidopsis thaliana sex1-3* mutant, which was phosphorylated retrospectively *in vitro* with an R1 protein.). R1 protein (Preparation B) was incubated in the presence of ATP labeled with 33P with starch isolated from leaves of an *Arabidopsis thaliana sex1-3* mutant. On completion of incubation, a total hydrolysis of the starch was carried out and the obtained hydrolysis products were separated by means of HPAE chromatography. As standard, glucose-6-phosphate and glucose-3-phosphate were added to the hydrolysis products before separation. The

hydrolysis products separated by means of HPAE chromatography were collected in individual fractions. The added glucose-6-phosphate eluted with fraction 15 and the added glucose-3-phosphate with fraction 17. The fractions obtained were subsequently investigated for the presence of radioactively labeled phosphate. The amount of ^{33}P labeled phosphate measured in the individual fractions, measured in cpm (counts per minute), which was introduced into the hydrolysis products of the phosphorylated starch by the OK1 protein or the R1 protein, is shown graphically.

Fig. 5 Demonstration of the autophosphorylation of the OK1 protein. Fig. 5 A) shows a Western blot. Fig. 5 B) shows the autoradiography of a denaturing (SDS) acrylamide gel. The same amounts of the same samples were applied to each of the two gels. The OK1 protein was incubated either with randomised radioactively labeled ATP or with ATP specifically radioactively labeled in the gamma position. On completion of incubation, the proteins were either heated to 30°C or 95°C, or incubated in 0.5 M NaOH or 0.5 M HCl respectively.

Fig. 6 Demonstration of the transfer of the beta-phosphate residue of ATP to starch in a reaction catalysed by an OK1 protein. Either ATP specifically labeled with ^{33}P in the gamma position or randomised ^{33}P ATP was used to phosphorylate starch, which had been phosphorylated *in vitro* by means of an R1 protein and isolated from leaves of an *Arabidopsis thaliana sex1-3* mutant, by means of an OK1 protein. No OK1 protein was added in any of the experiments designated as "control". Each test preparation was tested twice, independently from one another. The results of both tests are shown.

Fig. 7 Demonstration of the increase of the phosphorylation activity in phosphorylation reactions when R1 proteins and OK1 proteins are simultaneously involved in the reaction Presented is the incorporation of phosphate, starting from radioactively labeled ATP (randomised ^{33}P-ATP) in the

concerned starches by measuring the radioactivity (cpm] in the different starches. For this purpose, wheat starch was incubated in native form with R1 protein (Preparation 1-2, Preparation 1-2) or OK1 protein (Preparation 2), or in the form of *in vitro* phosphorylated wheat starch with OK1 protein (Preparation 3). Preparation 4 contained native wheat starch that was incubated simultaneously with R1 protein and OK1 protein. Each preparation was carried out in three repeats. The total of the respective preparations 1-2 and preparations 3 is presented for comparison.

Fig. 8 Demonstration of the increased activity through the cooperation of an R1 protein and an OK1 protein By means of an R1 protein, *in vitro* phosphorylated wheat starch was incubated for 10 minutes or 30 minutes respectively with purified R1 (Preparation 1) protein or purified OK1 protein (Preparation 2) in separate reaction preparations using randomised □/□-P^{33}ATP. In a parallel preparation, the same phosphorylated wheat starch was incubated simultaneously with an R1 protein and an OK1 protein (Preparation 3). For all reaction preparations, the amount of the phosphate bound to the starch in the respective reaction preparation was determined after completion of incubation by means of measurement in the scintillation counter. Also presented in the figure is the total for the reaction preparations for which only one of the two enzymes was introduced respectively for the phosphorylation reaction.

General methods

In the following, methods are described, which can be used for carrying the invention. These methods constitute specific embodiments of the present invention but do not restrict the present invention to these methods. The person skilled in the art knows that he can implement the invention in the same way by modifying the methods

described and/or by replacing individual parts of the methods by alternative parts of the methods.

1. Manufacture of protein extracts from plant tissues

a) Manufacture of protein extracts from plant tissues

Leaf material is frozen in liquid nitrogen immediately after harvesting and subsequently homogenised in the mortar under liquid nitrogen. The reduced leaf material is mixed with ca. 3.5-times the volume (referred to the weight of the leaf material used) of cold (4°C) binding buffer and broken down for 2 x 10 s with an Ultraturrax (maximum speed). After the first treatment with an Ultraturrax, the reduced leaf material is cooled on ice before the second treatment is carried out. The treated leaf material is then passed through a 100 µm nylon mesh and centrifuged for 20 min (50 ml centrifuge vessel, 20,000 xg, 4°C).

b) Precipitation of the proteins contained in the protein extracts

The supernatant obtained following centrifugation according to Step a) is removed and its volume determined. To precipitate proteins, ammonium sulphate is added continuously to the supernatant over a period of 30 minutes while stirring on ice down to a final concentration of 75% (weight/volume). The supernatant is subsequently incubated for a further hour on ice while stirring. The proteins precipitated from the supernatant are pellitised at 20,000 xg and 4°C for 10 min and the pellet subsequently absorbed in 5 ml of binding buffer, i.e. the proteins present in the pellet are dissolved.

c) Desalting of the precipitated proteins

The dissolved proteins are desalted by means of a PD10 column filled with Sephadex G25 (Amersham Bioscience, Freiburg, Prod. No. columns: 17-0851-01, Prod. No. Sephadex G25-M: 17-0033-01) at a temperature of 4°C, i.e. the ammonium sulphate used under Step b) for precipitation is also separated from the dissolved protein. The PD10 column is equilibrated with binding buffer before the proteins dissolved in

accordance with Step b) are applied. For this purpose, 5 ml of binding buffer are spread over the column five times in each case. Subsequently, 2.5 ml of the protein solution obtained in accordance with Step b) are added to each column before proteins are eluted from the column with 3.5 ml of binding buffer.

d) Determination of the protein concentration

The protein concentration is determined with a Bradford assay (Biorad, Munich, Prod. No. 500-0006, Bradford, 1976, Anal. Biochem. 72, 248-254).

e) Composition of the binding buffer [

Binding buffer:	50 mM	HEPES/NaOH (or KOH), pH 7.2
	1 mM	EDTA
	2 mM	Dithioerythritol (DTE)
	2 mM	Benzamidine
	2 mM	□-aminocaproic acid
	0.5 mM	PMSF
	0.02 %	Triton X-100

2. Isolation of leaf starch

a) Isolation of starch granules from plant tissues

Leaf material is frozen immediately after harvesting in liquid nitrogen. The leaf material is homogenised in portions in the mortar under liquid nitrogen and absorbed into a total of ca. 2.5-times the volume (weight/volume) of starch buffer. In addition, this suspension is again homogenised in the Waring blender for 20 s at maximum speed. The homogenate is passed through a nylon mesh (100 µm mesh width) and centrifuged for 5 minutes at 1,000 xg. The supernatant with the soluble proteins is discarded.

b) Purification of the starch isolated from the plant tissues

After removing the green material lying on top of the starch by rinsing off the green material with starch buffer, the pellet containing the starch obtained from Step a) is

absorbed in starch buffer and successively passed through nylon meshes with different mesh widths (in the order 60 µm, 30 µm, 20 µm). The filtrate is centrifuged using a 10 ml Percoll cushion (95% (v/v) Percoll (Pharmacia, Uppsala, Sweden), 5% (v/v) 0.5M HEPES-KOH pH7.2) (Correx tube, 15 min, 2,000 xg). The sediment obtained after this centrifugation is resuspended once in starch buffer and centrifuged again (5 min, 1,000 xg,).

c) Removal of the proteins bonded to the starch

Following Step b), starch granules are obtained, which contain proteins bonded to the starch. The proteins bonded to the surface of the starch granules are removed by incubating four times with 0.5 % SDS (sodium lauryl sulphate) for 10-15 minutes in each case at room temperature under agitation. Each washing step is followed by a centrifugation (5 min, 5,000 xg), in order to separate the starch granules from the respective wash buffer.

d) Purification of the starch that has been freed of proteins

The starch obtained from Step c), which has been freed from the proteins bonded to its surface, is subsequently removed by incubating four times with wash buffer for 10-15 minutes in each case at room temperature under agitation. Each washing step is followed by a centrifugation (5 min, 1,000 xg), in order to separate the starch granules from the respective wash buffer. These purification steps serve mainly to remove the SDS used in the incubations in Step c).

e) Determination of the concentration of isolated starch

The amount of starch isolated in Step d) is determined photometrically. After suitable dilution, the optical density of the starch suspension is measured against a calibration curve at a wavelength of 600 nm. The linear range of the calibration curve is located between 0 and 0.3 extinction units.

To produce the calibration curves, starch, for example isolated from leaves of an *Arabidopsis thaliana sex1-3* mutant, is dried under vacuum, weighed and absorbed in

a defined volume of water. The suspension so obtained is diluted with water in several steps in a ratio of 1 to 1 in each case until a suspension of ca. 5 µg of starch per ml of water is obtained. The suspensions obtained by the individual dilution steps are measured in the photometer at a wavelength of 600 nm. The absorption values obtained for each suspension are plotted against the concentration of starch in the respective suspension. The calibration curve obtained should follow a linear mathematical function in the range from 0 µg starch per ml of water to 0.3 µg starch per ml of water.

f) Storage of isolated starch

The starch can either be used directly without further storage for further tests, or stored in aliquots in 1.5 mL Eppendorf vessels at -20°C. Both the frozen starch and the non-stored, freshly isolated starch can be used, if required, for the methods described in the present invention relating to *in vitro* phosphorylation and/or bonding test, for example.

g) Composition of buffers used

1x starch buffer: 20 mM HEPES-KOH, pH 8.0
0.2 mM EDTA
0.5 % Triton X-100

Wash buffer: 50 mM HEPES/KOH, pH 7.2

3. Recombinant expression of an identified starch-phosphorylating protein

a) Manufacture of a bacterial expression vector containing a cDNA, which codes a starch-phosphorylating protein

The cDNA coding a starch-phosphorylating protein can be amplified, for example, using mRNA or poly-A-plus-mRNA from plant tissues as a "template", by means of a polymerase chain reaction (PCR). For this purpose, a reverse transcriptase is first used for the manufacture of a cDNA strand, which is complementary to an mRNA,

which codes a starch-phosphorylating protein, before the cDNA strand concerned is amplified by means of DNA polymerase. So-called "kits" containing substances, enzymes and instructions for carrying out PCR reactions are available for purchase (e.g. SuperScriptTM One-Step RT-PCR System, Invitrogen, Prod. No.: 10928-034). The amplified cDNA coding a starch-phosphorylating protein can subsequently be cloned in a bacterial expression vector, e.g. pDEST™17 (Invitrogen). pDEST™17 contains the T7 promoter, which is used to initiate the transcription of the T7-RNA-polymerase. Furthermore, the expression vector pDEST™17 contains a Shine Dalgarno sequence in the 5′-direction of the T7 promoter followed by a start codon (ATG) and by a so-called His tag. This His tag consists of six codons directly following one another, which each code the amino acid histidine and are located in the reading frame of the said start codon. The cloning of a cDNA coding a starch-phosphorylating protein in pDEST™17 is carried out in such a way that a translational fusion occurs between the codons for the start codon, the His tag and the cDNA coding a starch-phosphorylating protein. As a result of this, following transcription initiated on the T7 promoter, and subsequent translation, a starch-phosphorylating protein is obtained, which contains additional amino acids containing the His tag on its N-terminus.

However, other vectors, which are suitable for expression in microorganisms, can also be used for the expression of a starch-phosphorylating protein. Expression vectors and associated expression strains are known to the person skilled in the art and are also available for purchase from the appropriate dealer in suitable combinations.

b) Manufacture of expression clones in *Escherichia coli*

First of all, an appropriate transformation-competent *E. coli* strain, which chromosomally codes a T7-RNA polymerase, is transformed with the expression plasmid manufactured under Step a), and subsequently incubated overnight at 30°C on culture medium solidified with agar. Suitable expression strains are, for example, BL21 strains (Invitrogen Prod. No.: C6010-03, which chromosomally code a T7-RNA polymerase under the control of an IPTG-inducable promoter (lacZ)).

Bacteria colonies resulting from the transformation can be investigated using methods known to the person skilled in the art to see whether they contain the required expression plasmid containing a cDNA coding the starch-phosphorylating protein. At the same time, expression clones are obtained.

c) Expression of a starch-phosphorylating protein in *Escherichia coli*

First of all, a preliminary culture is produced. To do this, an expression clone obtained in accordance with Step b) is seeded in 30 ml Terrific Broth (TB medium) containing an antibiotic for selection on the presence of the expression plasmid, and incubated overnight at 30°C under agitation (250 rpm).

A main culture for the expression of a starch-phosphorylating protein is then produced. To do this, in each case, 1 liter Erlenmeyer flasks, each containing 300 ml of TB medium, pre-heated to 30°C, and an antibiotic for selection on the presence of the expression plasmid are each seeded with 10 ml of an appropriate pre-culture and incubated at 30°C under agitation (250 rpm) until an optical density (measured at a wavelength of 600 nm; OD_{600}) of ca. 0.8 is achieved.

If, for the expression of a starch-phosphorylating protein, an expression plasmid is used, in which the expression of the starch-phosphorylating protein is initiated by means of an inducable system (e.g. the expression vector pDEST™17 in BL21 *E. coli* strains, inducable by means of IPTG), then on reaching an OD_{600} of ca. 0.8, the inductor concerned (e.g. IPTG) is added to the main culture. After adding the inductor, the main culture is incubated at 30°C under agitation (250 rpm) until an OD_{600} of ca. 1.8 is achieved. The main culture is then cooled for 30 minutes on ice before the cells of the main culture are separated from the culture medium by centrifugation (10 minutes at 4,000 xg and 4°C).

4. Purification of a starch-phosphorylating protein

a) Breaking down of cells expressing a starch-phosphorylating protein

The cells obtained after centrifugation in Step c), Item 3 General Methods, are resuspended in lysis buffer. In doing so, ca. 4 ml lysis buffer are added to about 1 g of

cells. The resuspended cells are then incubated for 30 minutes on ice before they are broken down with the help of an ultrasonic probe (Baudelin Sonoplus UW 2070, Baudelin electronic, Berlin, settings: cycle 6, 70%, 1 minute) under continuous cooling by means of the ice. Care must be taken here to ensure that the cell suspension is not heated too much during the ultrasonic treatment. The suspension obtained after the ultrasonic treatment is centrifuged (12 minutes at 20,000 xg, 4°C) and the supernatant obtained after centrifugation is filtered using a filter with a pore size of 45 µm.

b) Purification of the starch-phosphorylating protein

If the starch-phosphorylating protein expressed in *E. coli* cells is a fusion protein with a His tag, then purification can take place with the help of nickel ions, to which the His tag bonds with greater affinity. To do this, 25 ml of the filtrate obtained in Step d) is mixed with 1 ml Ni-agarose slurry (Qiagen, Prod. No.: 30210) and incubated for 1 hour on ice. The mixture of Ni-agarose slurry and filtrate is subsequently spread over a polystyrene column (Pierce, Prod. No.: 29920). The product, which runs through the column, is discarded. The column is next washed by adding 8 ml of lysis buffer, whereby the product, which runs through the column, is again discarded. Elution of the starch-phosphorylating protein then takes place by fractionated addition to the column of 1 ml E1 buffer twice, followed by 1 ml E2 buffer once and subsequently 1 ml E3 buffer five times. The product, which runs through the column, which is produced by adding the individual fraction of the appropriate elution buffer (E1, E2, E3 buffer) to the column, is collected in separate fractions. Aliquots of these fractions are subsequently analysed by means of denaturing SDS acrylamide gel electrophoresis followed by Coomassie Blue colouring. The fractions, which contain the starch-phosphorylating protein in sufficient quantity and satisfactory purity, are purified and concentrated with the help of pressurised filtration at 4°C. Pressurised filtration can be carried out, for example, with the help of an Amicon cell (Amicon Ultrafiltration Cell, Model 8010, Prod. No.: 5121) using a Diaflo PM30 membrane

(Millipore, Prod. No.: 13212) at 4°C. Other methods known to the person skilled in the art can also be used for concentration however.

c) Composition of buffers used

Lysis buffer: 50 mM HEPES
300 mM NaCl
10 mM Imidazol
pH 8.0 (adjust with NaOH)
1 mg/ml Lysozyme (add immediately before using the buffer)
¼ tablet per 10 ml protease inhibitors completely EDTA free, (Roche product No.: 1873580) (add immediately before using the buffer)

Elution buffer E1: 50 mM HEPES
300 mM NaCl
50 mM Imidazol
pH 8.0 (adjust with NaOH)

Elution buffer E2: 50 mM HEPES
300 mM NaCl
75 mM Imidazol
pH 8.0 (adjust with NaOH)

Elution buffer E3: 50 mM HEPES
300 mM NaCl
250 mM Imidazol
pH 8.0 (adjust with NaOH)

5. Recombinant expression of an R1 protein

The recombinant expression of an R1 protein is described in the literature (Ritte et al., 2002, PNAS 99, 7166-7171; Mikkelsen et al., 2004, Biochemical Journal 377, 525-532), but can also be carried out in accordance with the methods relating to the recombinant expression of a starch expression of a starch-phosphorylating protein described above under Item 3. General Methods.

6. Purification of an R1 protein

The purification of an R1 protein is described in the literature (Ritte et al., 2002, PNAS 99, 7166-7171; Mikkelsen et al., 2004, Biochemical Journal 377, 525-532), but can also be carried out in accordance with the methods relating to the purification of a starch-phosphorylating protein described above under Item 4. General Methods if an R1 fusion protein, which contains a His tag, is produced by expression of R1 in E. coli cells.

7. In vitro manufacture of phosphorylated starch starting from non-phosphorylated starch

a) *In vitro* phosphorylation of non-phosphorylated starch

Starch, which does not contain starch phosphate (e.g., isolated from endosperm from maize or wheat plants respectively, or from leaves of *Arabidopsis thaliana sex1-3* mutants with the help of the methods described above under Item 2, General Methods), is mixed with R1 buffer and with purified R1 protein (ca. 0.25 µg R1 protein per mg starch) in order to produce a starch content of 25 mg per ml. This reaction preparation is incubated overnight (ca. 15 h) at room temperature under agitation. R1 bonded to the starch present in the reaction preparation is removed on completion of the reaction by washing four times with ca. 800 µl 0.5 % SDS in each case. Subsequently, the SDS still present in the *in vitro* phosphorylated starch is removed by washing five times with 1 ml wash buffer in each case. All washing steps are carried out at room temperature for 10 to 15 minutes under agitation. Each washing

step is followed by a centrifugation (2 min, 10,000 xg), in order to separate the starch granules from the respective SDS buffer.

b) Composition of buffers used

R1 buffer:	50 mM	HEPES/KOH, pH 7.5
	1 mM	EDTA
	6 mM	$MgCl_2$
	0.5 mM	ATP

Wash buffer: 50 mM HEPES/KOH, pH 7,2

8. Bonding of proteins to phosphorylated starch or non-phosphorylated starch

a) Isolation of P-starch protein complexes or non-phosphorylated starch protein complexes

Ca. 50 mg P-starch or ca. 50 mg non-phosphorylated starch respectively are resuspended in separate preparations in ca. 800 µl protein extract in each case. The protein concentration of the protein extracts should be ca. 4 mg to 5 mg per ml in each case. The incubation of the P-starch or non-phosphorylated starch with protein extracts is carried out at room temperature for 15 minutes at 4°C under agitation. On completion of the incubation, the reaction preparations are centrifuged out using a Percoll cushion (4 ml) (15 minutes, 3500 rpm, 4°C). After centrifugation, proteins that are not bonded to phosphorylated starch or P-starch will be found in the supernatant and can be removed with a Pasteur pipette. The supernatant is discarded. The sedimented pellet containing P-starch and non-phosphorylated starch, including the proteins bonded to the respective starches (P-starch protein complexes or non-phosphorylated starch protein complexes respectively), obtained after centrifugation is washed twice with 1 ml of wash buffer in each case (see above, General Methods under item 7.b) by incubating for 3 minutes at 4°C in each case under agitation. Each washing step is followed by a centrifugation (5 minutes, 8000 rpm, 4°C in a table

centrifuge, Hettich EBA 12R) in order to separate the P-starch or non-phosphorylated starch respectively from the wash buffer.

b) Dissolving the proteins bonded in the P-starch protein complexes or non-phosphorylated starch protein complexes respectively

The P-starch protein complexes or non-phosphorylated starch protein complexes respectively obtained in Step a) are resuspended in ca. 150 µl SDS test buffer and incubated at room temperature for 15 minutes under agitation. The P-starch or non-phosphorylated starch respectively is subsequently removed from the dissolved proteins by centrifugation (1 minute, 13,000 rpm, room temperature, Eppendorf table centrifuge). The supernatant obtained after centrifugation is centrifuged again in order to remove any residues of P-starch or non-phosphorylated starch respectively (1 minute, 13,000 rpm, room temperature, Eppendorf table centrifuge) and removed. As a result, dissolved proteins, which bond to the P-starch or non-phosphorylated starch respectively, are obtained.

c) Composition of buffers used

SDS test buffer:	187.5 mM	Tris/HCl pH 6.8
	6 %	SDS
	30 %	Glycerine
	~ 0.015 %	Bromophenol blue
	60 mM	DTE (add fresh!)

Percoll: Percoll is dialysed overnight against a solution consisting of and 25 mM HEPES / KOH, pH 7.0

9. Separation of proteins, which bond to P-starch and/or non-phosphorylated starch

The dissolved proteins obtained in Step c) under Item 8. General Methods relating to the bonding of proteins to P-starch or non-phosphorylated starch respectively are

incubated for 5 minutes at 95°C in each case and subsequently separated with the help of denaturing polyacrylamide gel electrophoresis. In doing so, an equal volume is applied to the acrylamide gel in each case for the dissolved proteins obtained by bonding to P-starch and for those obtained by bonding to non-phosphorylated starch. The gel obtained on completion of electrophoresis is stained at least overnight with colloidal Comassie (Roth, Karlsruhe, Roti-Blue Rod. No.: A152.1) and subsequently de-stained in 30 % methanol, 5 % acetic acid, or in 25% methanol.

10. Identification and isolation of proteins, which bond to P-starch and/or non-phosphorylated starch

a) Identification of proteins with increased bonding activity with respect to P-starch in comparison with non-phosphorylated starch

Proteins, which, after separation by means of acrylamide gel electrophoresis and subsequent visualisation by colouration (see above, Item 9. General Methods), exhibit an increased signal after bonding to P-starch in comparison with a corresponding signal after bonding to non-phosphorylated starch, have increased bonding activity with respect to P-starch in comparison with non-phosphorylated starch. By this means, it is possible to identify proteins, which have increased bonding activity with respect to P-starch in comparison with non-phosphorylated starch. Proteins, which have increased bonding activity with respect to P-starch in comparison with non-phosphorylated starch, are excised from the acrylamide gel.

b) Identification of amino acid sequence of proteins, which have increased bonding activity with respect to P-starch in comparison with non-phosphorylated starch

Proteins identified in accordance with Step a) are digested with trypsin, and the peptides obtained are analysed by means of MALDI-TOF to determine the masses of the peptides obtained. Trypsin is a sequence-specific protease, i.e. trypsin only splits proteins at a specified position when the proteins concerned contain certain amino acid sequences. Trypsin always splits peptide bonds when the amino acids arginine and lysine follow one another starting from the N-terminus. In this way, it is possible

to theoretically determine all peptides that would be produced following the trypsin digestion of an amino acid sequence. From the knowledge of the amino acids coding the theoretically determined peptides, the masses of the peptides, which are obtained after theoretical trypsin digestion, can also be determined. Databases (e.g. NCBInr http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm; Swissprot http://cbrg.inf.ethz.ch/Server/MassSearch.html), which contain information concerning the masses of peptides after theoretical trypsin digestion, can therefore be compared with the real masses of peptides of unknown proteins obtained with MALDI-TOF-MS. Amino acid sequences, which have the same peptide masses after theoretical and/or real trypsin digestion, are to be looked upon as being identical. The databases concerned contain both peptide masses of proteins, the function of which has already been shown, and also peptide masses of proteins, which up to now only exist hypothetically by derivation from amino acid sequences starting from nucleic acid sequences obtained in sequencing projects. The actual existence and the function of such hypothetical proteins has therefore seldom been shown and, if there is a function at all, then this is usually based only on predictions and not on an actual demonstration of the function.

Bands containing proteins identified in accordance with Step a) are excised from the acrylamide gel; the excised acrylamide piece is reduced and destained by incubating for approximately half an hour at 37°C in ca. 1 ml 60% 50mM NH_4HCO_3, 40% acetonitrile. The decolourising solution is subsequently removed and the remaining gel dried under vacuum (e.g. Speedvac). After drying, trypsin solution is added to digest the proteins contained in the gel piece concerned. Digestion takes place overnight at 37°C. After digestion, a little acetonitrile is added (until the acrylamide gel is stained white) and the preparation dried under vacuum (e.g. Speedvac). When drying is complete, just enough 5% formic acid is added to cover the dried constituents and incubated for a few minutes at 37°C. The acetonitrile treatment followed by drying is repeated once more. The dried constituents are subsequently absorbed in 0.1% TFA (triflouroacetic acid, 5 µl to 10 µl) and dripped onto a carrier in ca. 0.5 µl portions. Equal amounts of matrix (ε-cyano-4-hydroxy-cinnamic acid) are

also applied to the carrier. After crystallising out the matrix, the masses of peptides are determined by means of MALDI-TOF-MS-MS (e.g. Burker Reflex™ II, Bruker Daltonic, Bremen). With the masses obtained, databases are searched for amino acid sequences, which give the same masses after theoretical trypsin digestion. In this way, amino acid sequences can be identified, which code proteins, which preferably bond to phosphorylated alpha-1,4-glucans and/or which need P-alpha-1,4-glucans as a substrate.

11. Method for demonstrating starch-phosphorylating activity of a protein

a) Incubation of proteins with P-starch and/or non-phosphorylated starch

In order to demonstrate whether a protein has starch-phosphorylating activity, proteins to be investigated can be incubated with starch and radioactively labeled ATP. To do this, ca. 5 mg P-starch or ca. 5 mg non-phosphorylated starch is incubated with the protein to be investigated (0.01 µg to 5.0 µg per mg of added starch) in 500 µl phosphorylation buffer for 10 minutes to 30 minutes at room temperature under agitation. The reaction is subsequently stopped by the addition of SDS up to a concentration of 2% (weight/volume). The starch granules in the respective reaction mixture are centrifuged out (1 minute, 13,000 xg), and washed once with 900 µl of a 2 % SDS solution and four times each with 900 µl of a 2 mM ATP solution. Each washing step is carried out for 15 minutes at room temperature under agitation. After each washing step, the starch granules are separated from the respective wash buffer by centrifugation (1 min, 13,000 xg).

In addition, when carrying out an experiment to demonstrate starch-phosphorylating activity of a protein, further reaction preparations, which do not contain protein or contain inactivated protein, but which are otherwise treated in the same way as the reaction preparations described, should be processed as so-called controls.

b) Determination of the amount of phosphate residues incorporated in the P-starch and/or non-phosphorylated starch due to enzymatic activity

The starch granules obtained in accordance with Step a) can be investigated for the presence of radioactively labeled phosphate residues. To do this, the respective starch is resuspended in 100 µl of water and mixed with 3 ml of scintillation cocktail in each case (e.g. Ready Safe™, BECKMANN Coulter) and subsequently analysed with the help of a scintillation counter (e.g. LS 6500 Multi-Purpose Scintillation Counter, BECKMANN COULTER™).

c) Identification of proteins, which preferably use P-starch as a substrate

If a protein is incubated in separate preparations, once with P-starch and once with non-phosphorylated starch, in accordance with the method described under a), then, by comparing the values for the presence of starch phosphate obtained according to Step b), it can be determined whether the protein concerned has incorporated more phosphate in P-starch in comparison with non-phosphorylated starch. In this way, proteins can also be identified, which can introduce phosphate into P-starch but not into non-phosphorylated starch. This means proteins can be identified, which already require phosphorylated starch as a substrate for a further phosphorylation reaction.

d) Composition of buffers used

Phosphorylation buffer:

50 mM	HEPES/KOH, pH 7.5
1 mM	EDTA
6 mM	$MgCl_2$
0.01 to 0.5 mM	ATP
0.2 to 2 µCi per ml	randomised ^{33}P-ATP (alternatively, ATP, which contains a phosphate residue, which is specifically labeled in the gamma position, can also be used)

In conjunction with the present invention, the term "randomised ATP" is to be understood to mean ATP which contains labeled phosphate residues in both the gamma position as well as in the beta position (Ritte et al. 2002, PNAS 99, 7166-

7171). Randomised ATP is also described in the scientific literature as beta/gamma ATP. A method for manufacturing randomised ATP is described in the following.

i) Manufacture of randomised ATP

The method described here for manufacturing randomised ATP with the help of enzyme catalysed reactions is based on the following reaction mechanisms:

1st reaction step:

^{33}P-ATP + AMP + myokinase → ^{33}P-ADP + ADP

(Adenosine-P-P-^{33}P + Adenosine-P → Adenosine-P-P + Adenosine-P-^{33}P)

2nd reaction step

^{33}P-ADP + ADP + 2 PEP + Pyruvate kinase → ^{33}P-ATP + ATP + 2 Pyruvate

(Adenosine-P-P + Adenosine-P-^{33}P + 2 PEP → Adenosine-P-P-P + Adenosine-P-^{33}P-P + 2 Pyruvate)

The reaction equilibria lie on the product side but, in spite of this, this reaction produces a mixture consisting mainly of ^{33}P-ATP and some ^{33}P-ATP.

ii) Carrying out the 1st reaction step

ATP (100 µCi, 3000 Ci per mmol), which contains a phosphate residue labeled with ^{33}P in the gamma position (Hartmann Analytic, 10 µCi/µl), is incubated with 2 µl myokinase (AMP-phosphotransferase, from rabbit muscle; SIGMA, Prod. No.: M3003 3.8 mg/ml, 1,626 units/mg) in 90 µl randomising buffer for 1 hour at 37°C. The reaction is subsequently stopped by incubating for 12 minutes at 95°C before the reaction preparation is purified up by means of centrifugal filtration using a Microcon YM 10 filter (Amicon, Millipore Prod. No. 42407) at 14,000 xg for at least 10 minutes.

iii) Carrying out the 2nd reaction step

Two µl pyruvate kinase (see below for manufacture of an appropriate solution) and 3 µl 50 mM PEP (phosphoenolpyruvate) are added to the filtrate obtained in Step ii). This reaction mixture is incubated for 45 minutes at 30°C before the reaction is stopped by incubating at 95°C for 12 minutes. The reaction mixture is subsequently centrifuged (2 minutes, 12,000 rpm in an Eppendorf table centrifuge). The supernatant containing randomised ATP obtained after centrifugation is removed, aliquoted and can be stored at -20°C.

Manufacture of the pyruvate kinase solution

Fifteen µl pyruvate kinase (from rabbit muscle, Roche, Prod. No. 12815), 10 mg/ml, 200 units/mg at 25 °C) are centrifuged out, the supernatant discarded and the pellet absorbed in 27 µl pyruvate kinase buffer.

iv) Buffers used

Pyruvate kinase buffer:	50 mM	HEPES/KOH pH 7,5
	1 mM	EDTA
Randomising buffer:	100 mM	HEPES/KOH pH 7,5
	1 mM	EDTA
	10 %	Glycerol
	5 mM	$MgCl_2$
	5 mM	KCl
	0.1 mM	ATP
	0.3 mM	AMP

12. Demonstration of the autophosphorylation of a protein

In order to demonstrate whether a protein has auto-phosphorylating activity, proteins to be investigated can be incubated with radioactively labeled ATP. To do this, proteins to be investigated (50 µg to 100 µg) are incubated in 220 µl phosphorylation buffer (see above, Item 12 d), General Methods) for 30 minutes to 90 minutes at room

temperature under agitation. The reaction is subsequently stopped by the addition of EDTA up to a final concentration of 0.11 M. Ca. 2 µg to 4 µg of protein are separated with the help of denaturing polyacrylamide gel electrophoresis (7.5% acrylamide gel). The gel obtained after polyacrylamide gel electrophoresis is subjected to autoradiography. Proteins, which exhibit a signal in the autoradiography, carry a radioactive phosphate residue.

13. Identification of the C-atom positions of the glucose molecules of an alpha-1,4-glucan, into which phosphate residues are introduced by a starch-phosphorylating protein

Which C-atom positions of the glucose molecules of an alpha-1,4-Glucans are phosphorylated by a protein can be demonstrated in a controlled manner by hydrolysis of the phosphorylated glucans obtained by means of an appropriate protein *in vitro*, subsequent separation of the glucose monomers obtained after hydrolysis, followed by measurement of the phosphate incorporated by an appropriate protein in certain fractions of the glucose molecules.

a) Total hydrolysis of the alpha-1,4-glucans

Water suspensions containing alpha-1,4-glucan are centrifuged, the sedimented pellet subsequently resuspended in 0.7 M HCl (Baker, for analysis) and incubated for 2 hours at 95°C under agitation. On completion of incubation, the samples are briefly cooled and centrifuged (e.g. 2 minutes 10,000 xg). The supernatant obtained is transferred to a new reaction vessel and neutralised by the addition of 2 M NaOH (Baker, for analysis). If a pellet remains, it is resuspended in 100 µl of water and the quantity of labeled phosphate present therein is determined as a control.

The neutralised supernatant is subsequently centrifuged over a 10 kDa filter. By measuring an aliquot of the filtrate obtained, the quantity of labeled phosphate in the filtrate is determined with the help of a scintillation counter, for example.

b) Fractionation of the hydrolysis products and determination of the phosphorylated C-atom positions

The neutralised filtrates of the hydrolysis products obtained by means of Step a) can be separated (when using radioactively labeled ATP about 3,000 cpm) with the help of high-pressure anion exchange chromatography (HPAE), for example. The neutralised filtrate can be diluted with H_2O to obtain the volume required for HPAE. In addition, glucose-6-phosphate (ca. 0.15 mM) and glucose-3-phosphate (ca. 0.3 mM) are added to the appropriate filtrates in each case as an internal control. Separation by means of HPAE can be carried out, for example, with the help of a Dionex DX 600 Bio Lc system using a CarboPac PA 100 column (with appropriate pre-column) and a pulsed amperometric detector (ED 50). In doing so, before injecting the sample, the column is first rinsed for 10 minutes with 99% eluent C and 1% eluent D. A sample volume of 60 µl is then injected.

The elution of the sample takes place under the following conditions:

Flow rate: 1 ml per minute

Gradient: linearly increasing from 0 minutes to 30 minutes

	Eluent C:	Eluent D:
0 minutes	99%	1%
30 minutes	0%	100%
35 minutes	0%	100%
Run terminated		

The hydrolysis products eluted from the column are collected in individual fractions of 1 ml each. As, in each case, non-labeled glucose-3-phosphate (Ritte et al. 2002, PNAS 99, 7166-7171) and non-labeled glucose-6-phosphate (Sigma, Prod. No.: G7879) have been added to the injected samples of hydrolysis products as internal standards, the fractions, which contain either glucose-3-phosphate or glucose-6-phosphate, can be determined by means of pulsed amperometric detection. By measuring the amount of labeled phosphates in the individual fractions and subsequently comparing with the fractions, which contain glucose-3-phosphate or

glucose-6-phosphate, this can be used to determine those fractions, in which labeled glucose-6-phosphate or labeled glucose-3-phosphate is contained. The amount of labeled phosphate in the fraction concerned is determined. From the ratios of the amounts of glucose-3-phosphate to glucose-6-phosphate measured for labeled phosphate in the individual hydrolysis products, it can now be determined which C-atom position is preferably phosphorylated by an alpha-1,4-glucan phosphorylating enzyme.

c) Buffers used

Eluent C: 100 mM NaOH

Eluent D: 100 mM NaOH
500 mM sodium acetate

14. Transformation of rice plants

Rice plants were transformed in accordance with the methods described by Hiei et al. (1994, Plant Journal 6(2), 271-282).

15. Transformation of wheat plants

Wheat plants were transformed in accordance with the methods described by Becker et al. (1994, Plant Journal 5, 299-307).

16. Transformation of maize plants

Immature embryos of maize plants of the line A188 were transformed in accordance with the methods described by Ishida et al. (1996, Nature Biotechnology 14, 745-750).

17. Determination of the starch phosphate content

a) Determination of the C-6 phosphate content

The positions C2, C3 and C6 of the glucose units can be phosphorylated in the starch. For determining the C6-P content of the starch, 50 mg of starch is hydrolised in 500 µl of 0.7 M HCl for 4 hours at 95°C. The preparations are subsequently centrifuged for 10 minutes at 15500 g, and the supernatants are removed. Seven µl of the supernatants are mixed with 193 µl of imidazole buffer (100 MM imidazole, pH 7.4; 5 mM $MgCl_2$, 1 mM EDTA and 0.4 mM NAD). The measurement is carried out in the photometer at 340 nm. After establishing a base absorption, the enzyme reaction was started by adding 2 units of glucose-6-phosphate dehydrogenase (from Leuconostoc mesenteroides, Boehringer Mannheim). The change in absorption is directly proportional to the concentration of the G-6-P content of the starch.

b) Determination of the total phosphate content

The determination of the total phosphate content takes place according to the method by Ames (Methods in Enzymology VIII, (1996), 115-118).

Approximately 50 mg starch is mixed with 30 µl ethanolic magnesium nitrate solution and incinerated for three hours at 500°C in the muffle furnace. The residue is mixed with 300 µl 0.5 M hydrochloric acid and incubated 30 min at 60°C. An aliquot of 300 µl 0.5 M hydrochloric acid is subsequently refilled, added to a mixture of 100 µl of 10% ascorbic acid and 600 µl 0.42 % ammonium molybdate in 2 M sulfuric acid and incubated 20 minutes at 45°C.

c) Determination of the content of C-6 phosphate and C-3 phosphate

For determining the content of phosphate which is bound in the C-6 position and in the C-3 position of the glucose molecules of an alpha-1,4-glucan, the glucans concerned can be separated by means of HPAE after total hydrolysis according to the method presented under General Methods 13. The amounts of glucose-6-phosphate and glucose-3-phosphate can be determined by the integration of the individual peak areas obtained after HPEA separation. By comparing the peak areas obtained for glucose-6-phosphate and glucose-3-phosphate in unknown samples with the peak areas that are obtained with known amounts of glucose-6-phosphate and glucose-3-

phosphate after separation by means of HPEA, the amount of glucose-6-phosphate and glucose-3-phosphate can be determined in the sample to be investigated.

Examples

1. Isolation of a protein from *Arabidopsis thaliana*, which has increased bonding activity with respect to P-starch in comparison with non-phosphorylated starch

a) Manufacture of protein extracts from *Arabidopsis thaliana*

Protein extracts were manufactured from approximately 7 g of leaves (fresh weight) of *Arabidopsis thaliana* (Ecotype Columbia, Col-O) in accordance with the method described under Item 1, General Methods.

b) Isolation of starch granules from leaves of *sex1-3* mutants of *Arabidopsis thaliana*

Starch granules were isolated from approximately 20g (fresh weight) of leaves of a *sex1-3* mutant of *Arabidopsis thaliana* in accordance with the method described under Item 2, General Methods.

c) *In vitro* phosphorylation of starch isolated from a *sex1-3* mutant of *Arabidopsis thaliana* with purified R1 protein

About 30 mg of non-phosphorylated starch isolated from a *sex1-3* mutant of *Arabidopsis thaliana* was phosphorylated in accordance with the method described under Item 7, General Methods, by means of an R1 protein recombinantly expressed in *E. coli* and purified. The method described by Ritte et al. (2002, PNAS 99, 7166-7171) was used for expressing the R1 protein in *E. coli* and for subsequent purified.

d) Isolation of proteins, which bond to P-starch and/or non-phosphorylated starch

Protein extracts of *Arabidopsis thaliana*, obtained in accordance with Step a), were incubated and washed in a Preparation A with 50 mg of the *in vitro* phosphorylated

starch manufactured in accordance with Step c) using the method described under Item 8 a), General Methods.

Protein extracts of *Arabidopsis thaliana*, obtained in accordance with Step a), were incubated and washed in a second Preparation B with 50 mg of the non-phosphorylated starch manufactured in accordance with Step b) using the method described under Item 8 a), General Methods.

Subsequently, the proteins bonded to the P-starch of Preparation A and to the non-phosphorylated starch of Preparation B were dissolved in accordance with the method described under Item 8 b), General Methods.

In a third Preparation C, 50 mg of the *in vitro* phosphorylated starch manufactured in accordance with Step c) were incubated and washed using the method described under Item 8 a), General Methods. Preparation C contained no protein extracts, however.

e) Separation of the proteins obtained in accordance with Step d) by means of acrylamide gel electrophoresis

The proteins of Preparations A, B and C obtained in Step d) were separated by means of a 9% acrylamide gel under denaturing conditions (SDS) using the method described under Item 9, General Methods, and subsequently stained with Coomassie Blue. The stained gel is shown in Fig. 1. It can be clearly seen that a protein, which has a molecular weight of ca. 130 kDa in denaturing acrylamide gel referred to a protein standard marker (Trace M), preferably bonds to phosphorylated starch (Trace P) in comparison with non-phosphorylated starch (K).

f) Identification of the protein, which preferably bonds to P-starch in comparison with non-phosphorylated starch

The band of the protein with a molecular weight of ca. 130 kDa identified in Step e) was excised from the gel. The protein was subsequently released from the acrylamide as described under General Methods 10 b), digested with trypsin and the peptide masses obtained determined by means of MALD-TOF-MS. The so-called

"fingerprint" obtained by MALDI-TOF-MS was compared with fingerprints of theoretically digested amino acid molecules in databases (Mascot: http://www.matrixscience.com/search_form_select.html; ProFound: http://129.85.19.192/profound_bin/WebProFound.exe; PepSea: http://195.41.108.38/PepSeaIntro.html). As such a fingerprint is very specific to a protein, it was possible to identify an amino acid molecule. With the help of the sequence of this amino acid molecule, it was possible to isolate a nucleic acid sequence from *Arabidopsis thaliana* coding an OK1 protein. The protein identified with this method was designated A.t.-OK1. Analysis of the amino acid sequence of the OK1 protein from *Arabidopsis thaliana* showed that this deviated from the sequence that was present in the database (NP 198009, NCBI). The amino acid sequence shown in SEQ ID No 2 codes the A.t.-OK1 protein. SEQ ID No 2 contains deviations when compared with the sequence in the database (Acc.: NP 198009.1, NCBI). The amino acids 519 to 523 (WRLCE) and 762 to 766 (VRARQ) contained in SEQ ID No 2 are not in the sequence, which is present in the database (ACC.: NP 198009.1). Compared with Version 2 of the database sequence (Acc.: NP 198009.2), the amino acid sequence shown in SEQ ID NO 2 also contains the additional amino acids 519 to 523 (WRLCE).

2. Cloning of a cDNA, which codes the identified OK1 protein

The A.t.-OK1 cDNA was isolated with the help of reverse PCR using mRNA isolated from leaves of *Arabidopsis thaliana*. To do this, a cDNA Strand was synthesised by means of reverse transcriptase (SuperScript™ First-Strand Synthesis System for RT PCR, Invitrogen Prod. No.: 11904-018), which was then amplified using DNA polymerase (Expand High Fidelity PCR Systems, Roche Prod. No.: 1732641). The amplified product obtained from this PCR reaction was cloned in the vector pGEM®-T (Invitrogen Prod. No.: A3600). The plasmid obtained is designated A.t.-OK1-pGEM, the cDNA sequence coding the A.t.-OK1 protein was determined and is shown under SEQ ID NO. 1.

The sequence shown under SEQ ID NO 1 is not the same as the sequence, which is contained in the database. This has already been discussed for the amino acid sequence coding an A.t.-OKA.t.-OK1 protein.

Conditions used for the amplification of the cDNA coding the A.t.-OK1 protein

First strand synthesis:

The conditions and buffer specified by the manufacturer were used. In addition, the reaction preparation for the first strand synthesis contained the following substances:

3 µg	total RNA
5 µM	3'-Primer (OK1rev1: 5´-GACTCAACCACATAACACACAAAGATC)
0.83 µM	dNTP mix

The reaction preparation was incubated for 5 minutes at 75°C and subsequently cooled to room temperature.

The 1st strand buffer, RNase inhibitor and DTT were then added and incubated for 2 minutes at 42°C before 1 µL Superscript RT DNA polymerase was added and the reaction preparation incubated for 50 minutes at 42°C.

Conditions for the amplification of the first strand by means of PCR:

1 µL	of the reaction preparation of the first strand synthesis
0.25 µM	3'Primer (OK1rev2: 5´- TGGTAACGAGGCAAATGCAGA)
0.25 µM	5'Primer (OK1fwd2: 5´- ATCTCTTATCACACCACCTCCAATG)

Reaction conditions:

Step 1	95°C	2 min
Step 2	94°C	20 sec
Step 3	62°C	30 sec
Step 4	68°C	4 minutes
Step 5	94°C	20 sec
Step 6	56°C	30 sec
Step 7	68°C	4 minutes
Step 8	68°C	10 minutes

The reaction was first carried out in accordance with Steps 1 to 4. Ten repeats (cycles) were carried out between Step 4 and Step 2, the temperature of Step 3 being reduced by 0.67°C after each cycle. This was subsequently followed by the reaction in accordance with the conditions specified in Steps 5 to 8. Twenty-five repeats (cycles) were carried out between Step 7 and Step 5, the time of Step 7 being increased by 5 sec on each cycle. On completion of the reaction, the reaction was cooled to 4°C.

3. Manufacture of a vector for the recombinant expression of cDNA of the OK1 protein

Following amplification by means of PCR by using the plasmid A.t.-OK1-pGEM as a template using Gateway Technology (Invitrogen), the sequence coding the OK1 protein from *Arabidopsis thaliana* was next cloned in the vector pDONOR™ 201 (Invitrogen Prod. No.: 11798-014). Subsequently, the coding region of the OK1 protein from the vector obtained was cloned by sequence-specific recombination in the expression vector pDEST17™ (Invitrogen Prod. No.: 11803-014). The expression vector obtained was designated as A.t.-OK1-pDEST™17. The cloning resulted in a translational fusion of the cDNA coding the A.t-OK1 protein with the nucleotides present in the expression vector pDEST™17. The nucleotides originating from the vector pDEST™17, which are translationally fused with the cDNA coding the A.t.-OK1 protein, code 21 amino acids. These 21 amino acids include, amongst others, the start codon (ATG) and a so-called His tag (6 histidine residues directly after one another). After translation of these translationally fused sequences, this results in an A.t.-OK1 protein, which has the additional 21 amino acids coded by nucleotides originating from the vector at its N-terminus. The recombinant A.t.-OK1 protein resulting from this vector therefore contains 21 additional amino acids originating from the vector pDEST™17 at its N-terminus.

4. Heterological expression of the OK1 protein in E. coli

The expression vector A.t.-OK1-pDEST™17 obtained in accordance with Example 3 was transformed in the *E. coli* strain BL21 Star™ (DE3) (Invitrogen, Prod. No. C6010-03). A description of this expression system has already been given above (see Item 3, General Methods). Bacteria clones, containing the vector A.t.-OK1-pDEST™17, resulting from the transformation were next used to manufacture a preliminary culture, which was subsequently used for inoculating a main culture (see Item 3.c, General Methods). The preliminary culture and the main culture where each incubated at 30°C under agitation (250 rpm). When the main culture had reached an OD_{600} of ca. 0.8, the expression of the recombinant A.t.-OK1 protein was induced by the addition of IPTG (isopropyl-beta-D-thiogalactopyranoside) until a final concentration of 1 mM was achieved. After the addition of IPTG, the main culture was incubated at 30°C under agitation (250 rpm) until an OD_{600} of ca. 1.8 was achieved. The main culture was then cooled for 30 minutes on ice before the cells of the main culture were separated from the culture medium by centrifugation (10 minutes at 4,000 xg and 4°C).

5. Purification of the recombinantly expressed OK1 protein

The purification and concentration of the A.t.-OK1 protein from cells obtained in accordance with Example 4 was carried out using the method described under Item 4, General Methods.

6. Demonstration of starch-phosphorylating activity of the OK1 protein

The starch-phosphorylating activity of the A.t.-OK1 protein was demonstrated in accordance with the method described under Item 11, General Methods. In doing so, 5 µg of purified A.t.-OK1 protein manufactured in accordance with Example 5 was in each case incubated in a Preparation A with 5 mg of starch isolated from a *sex1-3* mutant of *Arabidopsis thaliana* in accordance with Example 1 b) and in a Preparation B with 5 mg of starch obtained by enzymatic phosphorylation in accordance with Example 1 c), in each case in 500 µl of phosphorylation buffer containing 0.05 mM radioactively (^{33}P) labeled, randomised ATP (in total 1,130,00 cpm, ca. 0.55 µCi) for 30 minutes at room temperature under agitation. A Preparation C was used as a

control, which was the same as Preparation B, except that it contained no OK1 protein, but was otherwise treated in the same way as Preparations A and B. Two tests, which were independent from one another, were carried out for all preparations (A, B, C).

Using a scintillation counter, the starches from Preparations A, B, and C were investigated for the presence of radioactively labeled phosphate (see Item 11 b), General Methods). The results are shown in Table 1 and in Fig. 3.

	Measured radioactivity [cpm]	
	Test 1	Test 2
Preparation A (non-phosphorylated starch + OK1)	42	47
Preparation B (phosphorylated starch + OK1)	7921	8226
Preparation C (phosphorylated starch without protein)	56	53

Table 1: Demonstration of starch-phosphorylating activity of the Ok1 protein

From the results obtained, it can be seen that the OK1 protein does not transfer phosphate groups from ATP to starch when non-phosphorylated starch is provided as a substrate, as the quota of phosphate groups transferred to non-phosphorylated starch by means of an OK1 protein, measured in cpm, does not exceed the quota of radioactively labeled phosphate groups in Preparation C (control). If, on the other hand, P-starch is provided as a substrate, the quota of radioactive phosphate groups, measured in cpm, which are transferred from ATP to P-starch, is significantly higher. From this, it can be seen that the OK1 protein requires P-starch as a substrate and that non-phosphorylated starch is not accepted as a substrate by the OK1 protein.

If the test described above is carried out with ATP specifically labeled in the gamma position with ^{33}P, then it is not possible to establish an incorporation of radioactively labeled phosphate in the starch. From this, it can be seen that the beta phosphate residue of ATP is transferred from an OK1 protein to starch. The results of such a test are shown in Fig. 6.

7. Demonstration of autophosphorylation

Autophosphorylation of the A.t.-OK1 protein was demonstrated by means of the methods described above (see Item 12, General Methods). Here, 50 µg of purified A.t.-OK1 protein were incubated with radioactively labeled, randomised ATP in 220 µl of phosphorylation buffer (see above, Item 12 d), General Methods) at room temperature for 60 minutes under agitation. Subsequently, 100 µl in each case were removed from the incubation preparations and transferred to four fresh reaction vessels. In reaction vessel 1, the reaction was stopped by the addition of 40 µl 0.11M EDTA. Reaction vessel 2 was incubated at 95°C for 5 minutes. HCl was added to reaction vessel 3 up to a final concentration of 0.5 M, and NaOH was added to reaction vessel 4 up to a final concentration of 0.5 M. Reaction vessels 3 and 4 were each incubated for 25 minutes at 30°C. Subsequently, 50 µl in each case were removed from reaction vessels 1, 2, 3 and 4, mixed with SDS test buffer and separated by means of SDS acrylamide gel electrophoresis (7.5% acrylamide gel). For this purpose, samples from the reaction vessels were applied to each of two identical acrylamide gels. One of the gels obtained on completion of electrophoresis was subjected to autoradiography, while the second gel was stained with Coomassie Blue.

In the gel stained with Coomassie Blue (see Fig. 2A)), it can be clearly seen that treatment with 0.5 M NaOH leads to a degradation of OK1 protein. The OK1 protein must therefore be described as unstable compared with NaOH. Incubations at 30°C, 95°C and with 0.5 M HCl show that the OK1 protein is relatively stable under the stated incubation conditions. This can be concluded from the fact that, under these incubation conditions, in each case approximately the same amounts of OK1 protein can be demonstrated in the gel concerned after colouring with Coomassie Blue.

In the autoradiography (see Fig. 2B)), it can be seen by comparison with the phosphorylated OK1 protein incubated at 30°C that an incubation of the phosphorylated OK1 protein at 95°C leads to a significant reduction in the phosphate, which has bonded to the OK1 protein. The bond between the phosphate residue and an amino acid of the OK1 protein must therefore be described as heat-unstable.

Furthermore, a slight reduction of the phosphate bonded to the OK1 protein can also be seen for the incubation with 0.5 M HCl and 0.5 M NaOH in comparison with phosphorylated OK1 protein incubated at 30°C. If the fact is taken into account that the quantity of OK1 protein in the autoradiography after treatment with 0.5 M NaOH is significantly less than in the samples treated with heat and acid on account of the instability of the OK1 protein compared with NaOH, then it can be concluded that the bond between the phosphate residue and an amino acid of the OK1 protein will be relatively stable with respect to bases. As the sample treated with acid contains approximately the same amounts of protein as the sample incubated at 30°C and at 95°C, and yet has a significantly lower signal in the autoradiography than the sample treated at 30°C, it must be assumed that acid incubation conditions also split the bond between a phosphate residue and an amino acid of the OK1 protein to a certain extent. An instability in the bond between a phosphate residue and an amino acid of the OK1 protein could therefore also be established in the tests carried out. At the same time, the instability with respect to acids is significantly less labeled than the instability with respect to heat.

Bonds between the amino acids histidine and phosphate are heat-unstable, acid-unstable but base-stable (Rosenberg, 1996, Protein Analysis and Purification, Birkhäuser, Boston, 242-244). The results described above are therefore an indication that a phosphohistidine is produced by the autophosphorylation of an OK1 protein.

If recombinantly expressed OK1 protein, as described above, is incubated with ATP specifically labeled with 33P in the gamma position, then no autophosphorylation can be detected. Fig. 5 A) shows the amount of protein in the respective reaction preparation that can still be demonstrated by means of Western blot analysis after the appropriate incubation steps. Fig. 5 B) shows an autoradiography of protein from the individual reaction preparations. It can be seen that, when ATP specifically labeled in the gamma position is used, no autophosphorylation of the OK1 protein takes place, whereas, when randomised ATP is used, autophosphorylation can be demonstrated. This means that when an OK1 protein is autophosphorylated, the phosphate residue

of the beta position of the ATP is covalently bound to an amino acid of the OK1 protein.

8. Demonstration of the C-atom positions, which are phosphorylated by an OK1 protein, of the glucose molecules of starch

a) Manufacture of phosphorylated starch

Phosphorylated starch was manufactured in accordance with Item 7, General Methods. To do this, 5 mg of non-phosphorylated starch, isolated from leaves of a *sex1-3* mutant of *Arabidopsis thaliana* were used in a Preparation A with 25 µg of purified A.t.-OK1 protein and, in a second Preparation B, 5 mg of *in vitro* phosphorylated starch originally isolated from leaves of a *sex1-3* mutant of *Arabidopsis thaliana* were used with 5 µg of purified R1 protein. The reaction was carried out in 500 µl of phosphorylation buffer in each case, which, in each case contained ^{33}P labeled ATP (ca. 2.5 x 10^6 cpm), by incubating at room temperature for 1 hour under agitation. In addition, a control preparation was used, which contained 5 mg of starch isolated from leaves of a *sex1-3* mutant of *Arabidopsis thaliana* and the said phosphorylation buffer, but no protein. The control preparation was treated in exactly the same way as Preparations A and B. The individual reactions were stopped by adding 125 µl 10% SDS in each case and washed with 900 µl in each case, once with 2% SDS, five times with 2 mM ATP and twice with H_2O. A centrifugation was carried out after each washing step (2 minutes in an Eppendorf table centrifuge at 13,000 rpm in each case). The starch pellets obtained were resuspended 1 ml H_2O in each case and 100 µl of each preparation were mixed after the addition of 3 ml of scintillation cocktail (Ready Safe™, BECKMANN) and subsequently measured with the help of a scintillation counter (LS 6500 Multi-Purpose Scintillation Counter, BECKMANN COULTER™).

The measurement gave the following results:

Control:	63 cpm/100 µL	630 cpm/1000 µL
Preparation A (OK1):	1351 cpm/100 µL	13512 cpm/1000 µL
Preparation B (R1):	3853 cpm/100 µL	38526 cpm/1000 µL

b) Total hydrolysis of the P-starch

The suspensions of Preparations A, B and C obtained in accordance with Step a) were centrifuged again (5 minutes in an Eppendorf table centrifuge at 13,000 rpm), the pellets obtained resuspended in 90 µl 0.7 M HCl (Baker, for analysis) and subsequently incubated for 2 hours at 95°C. Preparations A, B and C were then centrifuged again (5 minutes in an Eppendorf table centrifuge at 13,000 rpm), and the supernatant transferred to a new reaction vessel. Sedimented residues of the preparations were resuspended in 100 ml H_2O in each case and after the addition of 3 ml of scintillation cocktail (Ready Safe™, BECKMANN) were measured with the help of a scintillation counter (LS 6500 Multi-Purpose Scintillation Counter, BECKMANN COULTER™). Significant amounts of radioactivity could not be demonstrated in any of the residues, which means that all the hydrolysis products labeled with radioactive phosphate were located in the supernatant.

This was followed by neutralisation of the individual supernatants containing the hydrolysis products by the addition in each case of 30 µl 2 M NaOH (the amount of NaOH required for neutralisation was tested out in advance on blind samples). The neutralised hydrolysis products were placed on a 10 kDa Microcon filter, which had previously been rinsed twice with 200 µl H2O in each case, and centrifuged for ca. 25 minutes at 12,000 rpm in an Eppendorf table centrifuge. Ten µl were taken from the filtrate obtained (ca. 120 µl in each case) and, after the addition of 3 ml of scintillation cocktail (Ready SafeTM, BECKMANN), were measured with the help of a scintillation counter (LS 6500 Multi-Purpose Scintillation Counter, BECKMANN COULTERTM). The determination of the activity present in the individual preparations gave the following results:

Preparation A (OK1):	934 cpm/10 µL	11.208 cpm/120 µL	93 cpm/µl
Preparation B (R1):	2518 cpm/10 µL	30.216 cpm/120 µL	252 cpm/µl

c) Separation of the hydrolysis products

The hydrolysis products obtained in accordance with Step b) were separated by means of HPAE using a Dionex system under the conditions stated above (see

General Methods, Item 13 c)). The samples for separating the filtered supernatants of Preparations A and B obtained in accordance with Step b) were composed as follows:

Preparation A (OK1): 43 µl of the supernatant of Preparation A obtained in accordance with Step b) (equivalent to ca. 4,000 cpm), 32 µl H_2O, 2.5 µl 2.5 mM glucose-6-phosphate and 2.5 µl 5 mM glucose-3-phosphate (Volume = 80 µl).

Preparation B (R1): 16 µl of the supernatant of Preparation B obtained in accordance with Step b) (equivalent to ca. 4,000 cpm), 59 µl H_2O, 2.5 µl 2.5 mM glucose-6-phosphate and 2.5 µl 5 mM glucose-3-phosphate (Volume = 80 µl).

In each case, 60 µl, containing ca. 3,000 cpm, of the appropriate samples were injected for separation by means of HPAE. The HPAE was carried out in accordance with the conditions specified underGeneral Methods Item 13 c). After passing through the HPAE column, the elution buffer was collected in fractions, each of 1 ml. Collection of the fractions was begun 10 minutes after injecting the sample. Based on the signal received from the PAD detector used, the elution of glucose-6-phosphate was assigned to fraction 15 and the elution of glucose-3-phosphate to fraction 17. In each case, 500 µl of the individual fractions were mixed with 3 ml of scintillation cocktail (Ready SafeTM, BECKMANN) and subsequently measured with the help of a scintillation counter (LS 6500 Multi-Purpose Scintillation Counter, BECKMANN COULTERTM). The following measurements were obtained for the individual fractions:

	Total cpm per fraction	
	Preparation A (OK1):	Preparation B (R1):
Fr 13	8.7	3.3
Fr 14	13.1	32.2
Fr 15 (G6P)	207.3	1952.8
Fr 16	399.8	112.3
Fr 17 (G3P)	1749.2	801.6
Fr 18	196.7	17.3
Fr 19	6.7	18.9
Total	2581.5	2938.3
Deposit	3000.0	3000.0
Recovery	86.0%	97.9%

Table 4: Measured amounts of radioactivity [cpm] in individual fractions of hydrolysis products obtained by hydrolysis of starch phosphorylated by means of an OK1 protein or R1 protein.

The results are also shown graphically in Fig. 5.

After phosphorylation of starch catalysed by R1 protein, ca. 66% of the radioactively labeled phosphate, in terms of the total measured radioactive phosphate in the analysed fractions, eluted after hydrolysing the starch with the fraction, which contained glucose-6-phosphate as a standard, and ca. 27% with the fraction, which contained glucose-3-phosphate as a standard. After phosphorylation of starch catalysed by OK1 protein, ca. 67% of the radioactively labeled phosphate, in terms of the total measured radioactive phosphate in the analysed fractions, eluted after hydrolysing the starch with the fraction, which contained glucose-6-Phosphate as a standard, and ca. 8% with the fraction, which contained glucose-3-Phosphate as a standard. From this, it can be concluded that glucose molecules are preferably phosphorylated in the C-6 position by R1 proteins, whereas glucose molecules are preferably phosphorylated in the C-3 position by OK1 proteins.

9. Increase of the phosphorylation rate in simultaneous catalysis of the phosphorylation reaction by R1 proteins and OK1 proteins

a) *In vitro* phosphorylation of wheat starch

35 mg of wheat starch (Sigma, prod. no.: S-5127) per ml of reaction preparation were phosphorylated *in vitro* in accordance with the method described under Item 7, General Methods. Purified R1 protein in a concentration of 0.23 µg per mg of starch and ATP in a concentration of 25 µM was used for this purpose. The reaction time amounted to 1 hour at room temperature. In a parallel reaction preparation that contained randomised ^{33}P-ATP instead of ATP, the amount of the incorporated phosphate into the starch (0.0054 nmol per mg of starch) and the specific activity of the R1 protein used (0.41 nmol per (mg protein x minute) was determined. That the randomised ATP was used was not considered for this determination. The obtained values are thus lower that the actual existing values, because randomised ATP also contains phosphate residues labeled in the gamma position as well as phosphate residues labeled in the beta position.

b) Phosphorylation of native wheat starch and *in vitro* phosphorylated wheat starch by R1 and/or OK1 proteins

15 mg of wheat starch (Sigma, prod. no.: S-5127, or wheat starch phosphorylated *in vitro* in accordance with the method described under a)) was incubated with purified starch phosphorylated enzymes in 430 µl of the buffer containing 11 nmol of randomised ^{33}P-ATP (ca. 1.5×10^6 cpm) described under Item 11, General Methods, for one hour under agitation at room temperature. The individual reaction preparations contained the following protein and substrates:

	Protein	**Substrate**
Preparation 1-1	Purified R1 protein (3.4 µg)	Wheat starch (Sigma)
Preparation 1-2	Purified R1 protein (3.4 µg)	Wheat starch (Sigma)
Preparation 2	Purified OK1 protein (6.0 µg)	Wheat starch (Sigma)
Preparation 3	Purified OK1 protein (6.0 µg)	*In vitro* phosphorylated wheat starch
Preparation 4	Purified R1 protein (3.4 µg) Purified OK1 Protein (6.0 µg)	Wheat starch (Sigma)
Control	No protein	Wheat starch (Sigma)

Each of the preparations was carried out in three repeats in each case.

The treatment of the individual reaction preparations after one hour of reaction time and the determination of the incorporated amount of phosphate in the substrate concerned was carried out in accordance with the methods described under General Methods, Item 11.

The following results were obtained:

	Repeat 1 [cpm]	Repeat 2 [cpm]	Repeat 3 [cpm]
Preparation 1-1	11722	11584	11428
Preparation 1-2	12900	12204	11401
Preparation 2	-28	-21	-30
Preparation 3	2448	2281	2334
Preparation 4	17333	20337	16546
Total of Preparation 1-2 and Preparation 3	15348	14485	13735

Table 5: Measured quantity of radioactivity [cpm] in the individual repeats of the individual reaction preparations. The specified measurement values were determined by subtracting the measurement values of the associated controls from the actual measurement values in each case.

From the table it is clear that native wheat starch (Sigma, proc. No.: S-5127) forms no substrate for OK1 protein, whereas *in vitro* phosphorylated wheat starch can be phosphorylated from OK1 proteins. Furthermore, it can be seen that the activity is significantly higher than the total of the corresponding individual activities when an R1 protein and an OK1 protein are present simultaneously in the reaction mixture.

c) Phosphorylation of *in vitro* phosphorylated wheat starch by R1 and/or OK1 proteins

Phosphorylated wheat starch was manufactured in accordance with the method described under a). The amount of phosphate bound to starch amounted to 0.0048 mg of phosphate per mg of starch. That the randomised ATP was used was not considered for this determination, as also described under a).

15 mg of *in vitro* phosphorylated wheat starch was incubated with starch phosphorylating enzymes in accordance with the methods described under b). The individual reaction preparations contained the following protein and substrates:

	Protein	**Substrate**
Preparation 1: R1	Purified R1 protein (3.4 µg)	*In vitro* phosphorylated wheat starch
Preparation 2: OK1	Purified OK1 protein (5.2 µg)	*In vitro* phosphorylated wheat starch
Preparation 3: R1+OK1	Purified R1 protein (3.4 µg) Purified OK1 Protein (5.2 µg)	*In vitro* phosphorylated wheat starch

Each of the preparations was carried out in two repeats in each case.

A sample was stopped after 0, 10 and 30 minutes of incubation time for each reaction preparation, and the amount of phosphate incorporated under the respective reaction conditions was determined in accordance with the method described under General methods, Item 11.

As a control, native wheat starch was incubated with R1 protein in the presence of ATP not radioactively labeled. Subsequently, the reaction mixture was added to randomised ^{33}P-ATP and buffer before the reaction was stopped.

The following results were obtained:

	0 minutes reaction time	10 minutes reaction time	30 minutes reaction time
Preparation 1	0 cpm	2378 cpm	7543 cpm
Preparation 2	0 cpm	2032 cpm	3005 cpm
Preparation 3	0 cpm	7570 cpm	16245 cpm
Total of Preparation 1 and Preparation 2	0 cpm	4410 cpm	10548 cpm

Table 6: Measured quantity of radioactivity [cpm] in the individual repeats of the individual reaction preparations. The specified measurement values were determined by subtracting the measurement values of the associated controls from the average value of two independent measurements.

10. Identification of an OK1 protein in rice

Using the methods described under Items 1 to 13, General Methods, it was also possible to identify a protein from *Oryza sativa* (variety M202), which transfers a phosphate residue from ATP to P-starch. The protein was designated as O.s.-OK1. Non-phosphorylated starch is not used by the O.s.-OK1 protein as a substrate, i.e. the O.s.-OK1 protein also does not need P-starch as a substrate. The nucleic acid sequence defining the identified O.s.-OK1 protein is shown under SEQ ID NO 3 and the amino acid sequence coding the O.s.-OK1 protein is shown under SEQ ID NO. 4. The amino acid sequence coding the O.s.-OK1 protein shown under SEQ ID NO 4 has an identity of 57% with the amino acid sequence coding the A.t.-OK1 protein shown under SEQ ID NO 2. The nucleic acid sequence coding the O.s.-OK1 protein

shown under SEQ ID NO 3 has an identity of 61% with the nucleic acid sequence coding the A.t.-OK1 protein shown under SEQ ID NO 1.

Manufacture of the plasmid pMI50 containing the nucleic acid sequence coding an OK1 protein from *Oryza sativa*

The vector pMI50 contains a DNA fragment, which codes the complete OK1 protein from rice of the variety M202.

The amplification of the DNA from rice was carried out in five sub-steps.

1. The part of the open reading frame from position 11 to position 288 of the sequence specified under SEQ DIE NO 3 was amplified with the help of reverse transcriptase and polymerase chain reaction using the synthetic oligonucleotides Os_ok1-R9 (GGAACCGATAATGCCTACATGCTC) and Os_ok1-F6 (AAAACTCGAGGAGGATCAATGACGTCGCTGCGGCCCCTC) as a primer on RNA of immature rice seeds. The amplified DNA fragment was cloned in the vector pCR2.1 (Invitrogen catalogue number K2020-20). The obtained plasmid was designated as pML123.

2. The part of the open reading frame from position 250 to position 949 of the sequence specified under SEQ DIE NO 3 was amplified with the help of reverse transcriptase and polymerase chain reaction using the synthetic oligonucleotides Os_ok1-F4 (CCAGGTTAAGTTTGGTGAGCA) and Os_ok1-R6 (CAAAGCACGATATCTGACCTGT) as a primer on RNA of immature rice seeds. The amplified DNA fragment was cloned in the vector pCR2.1 (Invitrogen catalogue number K2020-20). The obtained plasmid was designated as pML120.

3. The part of the open reading frame from position 839 to position 1761 of the sequence specified under SEQ DIE NO 3 was amplified with the help of reverse transcriptase and polymerase chain reaction using the synthetic oligonucleotides Os_ok1-F7 (TTGTTCGCGGGATATTGTCAGA) and Os_ok1-R7 (GACAAGGGCATCAAGAGTAGTATC) as a primer on RNA of immature rice seeds.

The amplified DNA fragment was cloned in the vector pCR2.1 (Invitrogen catalogue number K2020-20). The obtained plasmid was designated as pML121.

4. The part of the open reading frame from position 1571 to position 3241 of the sequence specified under SEQ DIE NO 3 was amplified with the help of reverse transcriptase and polymerase chain reaction using the synthetic oligonucleotides Os_ok1-F8 (ATGATGCGCCTGATAATGCT) and Os_ok1-R4 (GGCAAACAGTATGAAGCACGA) as a primer on RNA of immature rice seeds. The amplified DNA fragment was cloned in the vector pCR2.1 (Invitrogen catalogue number K2020-20). The obtained plasmid was designated as pML119.

5. The part of the open reading frame from position 2777 to position 3621 was amplified with the help of polymerase chain reaction using the synthetic oligonucleotides Os_ok1-F3 (CATTTGGATCAATGGAGGATG) and Os_ok1-R2 (CTATGGCTGTGGCCTGCTTTGCA) as a primer on genomic DNA of rice. The amplified DNA fragment was cloned in the vector pCR2.1 (Invitrogen catalogue number K2020-20). The obtained plasmid was designated as pML122.

The cloning together of the sub-parts of the open reading frame of OK1 was carried out as follows.

A 700 base pair long *Apa*I fragment of pML120, containing part of the open reading frame of OK1, was cloned in the *Apa*I site of pML121. The obtained plasmid was designated as pMI47.

A 960 base pair long fragment containing the areas of vectors from pML120 and pML123 coding for OK1 was amplified by means of polymerase chain reaction. In doing so, the primers Os_ok1-F4 (see above) and Os_ok1-R9 (see above), each in a concentration of 50 nm, and the primers Os_ok1-F6 and Os_ok1-R6, each in a concentration of 500 nm, were used. The amplified DNA fragment was cloned in the vector pCR2.1 (Invitrogen catalogue number K2020-20). The obtained plasmid was designated as pMI44.

An 845 base pair long fragment of pML122 was reamplified for introducing an XhoI site after the stop codon with the primers Os_ok1-F3 (see above) and Os_ok1-R2Xho

(AAAACTCGAGCTATGGCTGTGGCCTGCTTTGCA) and cloned in the vector pCR2.1 (Invitrogen catalogue number K2020-20). The obtained plasmid was designated as t pMI45.

A 1671 base pair long fragment containing part of the open reading frame of OK1 was obtained from pML119 by digesting with the restriction enzymes *Spe*I and *Pst*I. The fragment was cloned in pBluescript II SK+ (Genbank Acc.: X52328). The obtained plasmid was designated as pMI46.

A 1706 base pair long fragment containing part of the open reading frame of OK1 was excised with the restriction enzymes *Spe*I and *Xho*I from pMI46 and cloned in the vector pMI45, which had been excised with the same restriction enzymes. The obtained plasmid was designated as pMI47.

A 146 base pair long fragment containing part of the open reading frame of OK1 was excised with the restriction enzymes *Afl*II/*Not*I from pMI43 and cloned in the vector pMI44, which had been excised with the same restriction enzymes. The obtained plasmid was designated as pMI49.

A 1657 base pair long fragment containing part of the open reading frame of OK1 was excised with the restriction enzymes *Not*I and *Nar*I from the vector pMI49 and cloned in the vector pMI47, which had been excised with the same restriction enzymes. The plasmid obtained was designated as pMI50 and contains the whole coding region of the OK1 protein identified in rice.

11. Manufacture of an antibody, which specifically detects an OK1 protein

As an antigen, ca. 100 µg of purified A.t.-OK1 protein was separated by means of SDS gel electrophoresis, the protein bands containing the A.t.-OK1 protein excised and sent to the company EUROGENTEC S.A. (Belgium), which carried out the manufacture of the antibody under contract. Next, the preimmune serums of rabbits were investigated to see whether they would already detect a protein from an A. t. total extract before immunisation with recombinant OK1. The preimmune serums of two rabbits detected no proteins in the range 100-150 kDa and were thus chosen for immunisation. 4 injections of 100 µg of protein (day 0, 14, 28, 56) were given to each

rabbit. 4 blood samples were taken from each rabbit: (day 38, day 66, day 87 and the final bleeding). Serum obtained after the first bleeding already showed a specific reaction with OK1 antigen in Western blot. However, in all further tests, the last bleeding of a rabbit was used.

12. Manufacture of transgenic maize plants that have an increased activity of an OK1 protein and an R1 protein

a) Manufacture of a construct for the transformation of maize plants that overexpress an R1 protein

The plasmid pMZ12 was used as a starting plasmid for the manufacture of a plasmid, which was used for the transformation of maize plants. This plasmid contains the *ColE1* origon of the plasmid pBR322) Bolivar et al, 1977, Gene 2, 95-113) and a bacterial selection marker that facilitates a resistance against the antibiotic gentamycin (Wohlleben et al., 1989 MGG 217, 202-208). Furthermore, this plasmid contains a right and a left T-DNA border sequence. Between these T-DNA border sequences the plasmid contains a *bar* gene from *Streptomyces hygroscopicus* (White et al., 1990, NAR 18, 1062; EMBL Acc.: X17220), which facilitates resistance against the herbicide glufosinate. The expression of the bar gene is initiated through the promoter of the *actin* gene from rice (McElroy et al., 1990, Plant Cell 2, 163.171). To stabilise of the expression of the *bar* gene, the 1st intron of the *actin* gene from rice is inserted between the *actin* promoter and the sequence coding the bar protein (McElroy et al., 1990, Plant Cell 2, 163.171). The polyadenylation signal of the nopaline synthase gene from *Agrobacterium tumefaciens* follows after the sequence coding the *bar* protein (Depicker et al., 1982 J Mol. Appl. Gent. 1, 561-573).

The ubiquitin promoter from *Zea mays* was inserted into the plasmid pMZ12 between the left and right T-DNA border sequence (Christensen et al., 1992, Plant Mol. Bio 18, 675-689), followed by the 1st intron of the ubiquitin gene from *Zea mays* (Christensen et al., 1992, Plant Mol. Bio 18, 675-689), followed by the coding sequence of the R1 gene from *Solanum tuberosum* (see SEQ ID NO 10), followed by the polyadenylation signal of the nopaline synthase gene from *Agrobacterium tumefaciens* (Depicker et

al., 1982, J Mol. Appl. Gent. 1, 561-573). The obtained plasmid was designated as pHN3-146.

b) Transformation of maize plants with the plasmid pHN3-146

Premature embryos of maize plants were isolated ten days after pollination and transformed with the help of *Agrobacterium tumefaciens* containing the plasmid pHN3-146 as a cointegrate in accordance with the methods described by Ishida et al. (1996, Nature Biotechnology 14, 745-750). So-called T0 plants resulting from this transformation were raised in the greenhouse.

c) Identification of maize plants that have an increased expression of the S.t.-R1 protein from *Solanum tuberosum*

By means of quantitative RT PCR analysis, it was possible to identify plants, which had an expression of mRNA coding the S.t.-R1 protein.

d) Manufacture of the plasmid pUbi-A.t.-OK1

First, the plasmid pIR96 was manufactured. The plasmid pIR96 was obtained by cloning a synthetic piece of DNA consisting of the two oligonucleotides X1 (TGCAGGCTGCAGAGCTCCTAGGCTCGAGTTAACACTAGTAAGCTTAATTAAGAT ATCATTTAC) and X2 (AATTGTAAATGATATCTTAATTAAGCTTACTAGTGTTAACTCGAGCCTAGGAGCT CTGCAGCCTGCA) in the vector pGSV71 excised with *SdaI* and *MunI*. The plasmid obtained was excised with *SdaI,* and the protruding 3'-ends were smoothed with T4 DNA polymerase. The plasmid obtained was excised with SdaI, the protruding 3'-ends smoothed with T4 DNA polymerase and a smoothed HindIII / SphI fragment from pBinAR (Höfgen and Willmitzer, 1990, Plant Science 66, 221-230) with a size of 197 base pairs, containing the termination signal of the octopine synthase gene from *Agrobacterium tumefaciens*, was inserted. The obtained plasmid was designated as pIR96.

pGSV71 is a derivative of the plasmid pGSV7, which derives from the intermediate vector pGSV1. pGSV1 constitutes a derivative of pGSC1700, the construction of which has been described by Cornelissen and Vanderwiele (Nucleic Acid Research 17, (1989), 19-25). pGSV1 was obtained from pGSC1700 by deletion of the carbenicillin resistance gene and deletion of the T-DNA sequences of the TL-DNA region of the plasmid pTiB6S3.

pGSV7 contains the replication origin of the plasmid pBR322 (Bolivar et al., Gene 2, (1977), 95-113) as well as the replication origin of the Pseudomonas plasmid pVS1 (Itoh et al., Plasmid 11, (1984), 206). pGSV7 also contains the selectable marker gene aadA, from the transposon Tn1331 from *Klebsiella pneumoniae*, which gives resistance against the antibiotics spectinomycin and streptomycin (Tolmasky, Plasmid 244 (3), (1990), 218-226; Tolmasky and Crosa, Plasmid 29(1), (1993), 31-40).

The plasmid pGSV71 was obtained by cloning a chimeric bar gene between the border regions of pGSV7. The chimeric bar gene contains the promoter sequence of the cauliflower mosaic virus for initiating the transcription (Odell et al., Nature 313, (1985), 180), the bar gene from Streptomyces hygroscopicus (Thompson et al., 1987, EMBO J. 6, 2519-2523) and the 3'-untranslated area of the nopaline synthase gene of the T-DNA of pTiT37 for terminating the transcription and polyadenylation. The bar gene provides tolerance against the herbicide glufosinate ammonium.

A 1986 base pair long fragment containing the promoter of the polyubiquitin gene from maize (EMBLK Acc.: 94464, Christensen et al., 1992, Plant Mol. Biol. 18: 675-689) was cloned as a *Pst*I fragment in pBluescript II SK+. The obtained plasmid was designated as pSK-ubq.

The plasmid A.t.-OK1-pGEM was excised with the restriction enzymes *Bsp*120I, smoothed with T4-DNA-polymerase and excised with *Sac*I. The DNA fragment coding the OK1 protein from *Arabidopsis thaliana* was cloned in the plasmid pSK-ubq, which was excised with *Sma*I and *Sac*I. The obtained plasmid was designated as pSK-ubq-ok1.

A fragment which contained the ubiquitin promoter from maize and the total open reading frame for the A.t.-OK1 protein from *Arabidopsis thaliana* was isolated from

the plasmid pSK-ubq-ok1. To do this, the plasmid was excised with the restriction enzyme *Asp*718I, the ends refilled with T4 DNA polymerase and excised with *Sda*I. The fragment with a size of 5799 base pairs that was obtained was cloned in the plasmid pIR96 excised with *Eco*RV and *Pst*I. The obtained plasmid from this cloning was designated as pUbi-A.t.-OK1.

e) Transformation of maize plants with the plasmid pUbi-A.t.-OK1

Ten days after pollination, immature embryos of maize plants were isolated and transformed with the help of *Agrobacterium tumefaciens* containing the plasmid pUbi-A.t.-OK1 as a cointegrate according to the methods described by Ishida et al. (1996, Nature Biotechnology 14, 745-750). So-called T0 plants resulting from this transformation were raised in the greenhouse.

f) Identification of maize plants that have an increased expression of the A.t.-OK1 protein from *Arabidopsis thaliana*

By means of quantitative RT PCR analysis, it was possible to identify plants that had an expression of mRNA coding the A.t.-OK1 protein.

g) Production of homozygotic plants that have an increased expression of the S.t.-R1 protein or of the A.t.-OK1 protein.

For T1 plants that have an expression of the S.t.-R1 protein or of the A.t.-OK1 protein, seeds of the individual plants were harvested, and in each case ca. 30 seeds per plant were laid out again and cultivated in the greenhouse. Plants of this T1 generation were sprayed in the three-leaf stage with a solution containing 0.5% Basta®. Only those groups of T1 plants for which ca. 25% of the 30 cultivated plants in each case died off after spraying with the Basta® solution were followed further, because these plants are those for which the integration of the related T-DNA of the plasmid pHN3-146 or pUbi-A.t.-OK1 is present in a locus in the genome. Genomic DNA was isolated from leaf material from the ca. 75% of the plants that survived the spraying with Basta® solution and investigated in each case for the number of copies

present in case by means of Invader® technology (Pielberg et al. 2003, Genome Res.;13, 2171-2177). The T1 plants within a group of offspring of a T0 plant that showed a signal approximately twice as strong as the remaining offspring of the same T0 plant in an analysis by means of Invader® technology are homozygotic with respect to the locus at which the T-DNA of the related plasmid is integrated. If approximately 30% of the offspring of a T0 plant that survived the treatment with Basta® solution show a signal approximately twice as strong in the analysis by means of Invader technology, in comparison with the remaining ca. 70% of the offspring of the same T0 plant, then this is a further indication that the integration of the T-DNA is at a single locus.

h) Production of plants that have both an increased expression of the S.t.-R1 protein as well as an increased expression of the A.t.-OK1 protein

T1 plants that have an increased expression of an S.t.-R1 protein and that are homozygotic with respect to the integration of the T-DNA of the plasmid pHN3-146 according to the analysis described under g), and in which the integration exists at a locus in the genome of the plant, are crossed with T1 plants that have an increased expression of an A.t.-OK1 protein and are homozygotic with respect to the integration of the T-DNA of the plasmid pUbi-A.t.-OK1, and in which the integration exists at a locus in the genome of the plant. The offspring of these crosses have both an increased expression of the S.t.-R1 protein as well as an increased expression of the A.t.-OK1 protein.

i) Analysis of the grains of transgenic maize plants and starches synthesised from them

Starches were isolated from the grains of the related maize plants resulting from the crosses described under h). The starch from grains that have an increased expression of the S.t.-R1 protein and an increased expression of the A.t.-OK1 protein, contains more phosphate covalently bound to the starch, than does starch isolated from untransformed wild type plants.

Starch isolated from grains that have an increased expression of the S.t.-R1 protein and an increased expression of the A.t.-OK1 protein, likewise contains more phosphate covalently bound to the starch than does starch isolated from plants that have only an increased expression of the S.t.-R1 protein or only an increased expression of the A.t.-OK1 protein.

13. Manufacture of transgenic wheat plants that have an increased expression of an OK1 protein and an R1 protein

a) Manufacture of transgenic wheat plants that overexpress an R1 protein

The manufacture of wheat plants, which have an increased expression of the R1 protein from potato, were described in WO 02 34923. The plants described there were partially used as a starting material for the manufacture of plants that have an increased expression of an OK1 protein and an R1 protein.

b) Manufacture of a plasmid for the transformation of wheat plants that overexpress an OK1 protein

pMCS5 (Mobitec, www.mobitec.de) was digested with *BglII* and *BamHI* and relegated. The obtained plasmid was designated as pML4.

The *nos*-terminator from *Agrobacterium tumefaciens* (Depicker et al., 1982, Journal of Molecular and Applied Genetics 1: 561-573) was amplified with the primers P9 (ACTTCTgCAgCggCCgCgATCgTTCAAACATTTggCAATAAAgTTTC) and P10 (TCTAAgCTTggCgCCgCTAgCAgATCTgATCTAgTAACATAgATgACACC) (25 cycles, 30 sec 94°C, 30 sec 58°C, 30 sec 72°C), digested with *HindIII* and *PstI*, and cloned in the plasmid pML4 excised with the same enzyme. The obtained plasmid was designated as pML4-nos. A 1986 base pair long fragment containing the promoter of the polyubiquitin gene from maize (Genbank Acc.: 94464, Christensen et al., 1992, Plant Mol. Biol. 18: 675-689) and the first intron of the same gene shortened by digestion with *ClaI* and religation was cloned. The obtained plasmid was designated as pML8.

The total open reading frame of the OK1 from *Arabidopsis thaliana* was cloned in the plasmid pML8. For this purpose the corresponding fragment was excised with *Bsp120/NotI* from A.t.-OK10pGEM and ligated in sense orientation into the *NotI* site. With the restriction enzymes *AvrII* and *SwaI*, a fragment for the transformation of wheat plants can be excised from the obtained vector pML8-A.t.-OK1, which contains the promoter of the polyubiquitin gene from maize, the total open reading frame of OK1 from *Arabidopsis thaliana* and the *nos*-terminator from *Agrobacterium tumefaciens*.

c) Manufacture of a plasmid for the production of wheat plants that overexpress an R1 protein

A plasmid was manufactured in which the DNA fragment, which coded for the complete R1 protein from potato, lies between two detection sites for the restriction enzyme *Pac*I. For this purpose, the *Multiple Cloning Site* from the plasmid pBluescript II SK+ was amplified with the help of the polymerase chain reaction and both oligonucleotides MCS1-1 (TTTTTGCGCGCGTTAATTAACGACTCACTATAGGGCGA) and MCS1-2 (TTTTTGCGCGCTTAATTAACCCTCACTAAAGGGAACAAAAG), excised with the restriction enzyme *Bss*HII, and cloned in the dephosphorylated vector pBluescript II SK+ (Invitrogen) excised with *Bss*HII. The obtained plasmid was designated as pSK-Pac.

A *Not*I fragment was cloned in the vector pSK-Pac, which was obtained from the clone pRL2 (WO 9711188). The *Not*I fragment contains the total open reading frame for the R1 protein from potato. The obtained plasmid was designated as pIR1.

A fragment, which contained the ubiquitin promoter and the shortened first intron, and cloned in the *Eco*RV site of the plasmid, was excised with the restriction enzymes *Eco*RV and *Sma*I from pSK-ubq (see above). In the obtained plasmid, a *Pac*I fragment was cloned from pIR1 in sense orientation to the promoter, which contains the total open reading frame coding for the R1 protein from potato. The obtained plasmid was designated as pML82.

d) Transformation of wheat plants for overexpression of an OK1 protein

Wheat plants of the Florida variety were transformed by means of the biolistic method in accordance with the method described by Becker et al. (1994, Plant Journal 5, 299-307) with a fragment excised from an agarose gel, which was excised with the restriction enzymes *AvrII* and *SwaI* from the plasmid pML8-A.t.-OK1 and contained the promoter of the polyubiquitin gene from maize, the total open reading frame of OK1 from *Arabidopsis thaliana* and the *nos*-terminator from *Agrobacterium tumefaciens* together with the plasmid pGSV71. The obtained plants were designated as TA-OK1.

e) Transformation of wheat plants for overexpression of an R1 protein

Wheat plants of the Florida variety were transformed with the plasmid pML82 by means of the biolistic method in accordance with the method described by Becker et al. (1994, Plant Journal 5, 299-307). The obtained plants were designated as TA-R1.

f) Cotransformation of wheat plants for the overexpression of an OK1 protein and an R1 protein

Wheat plants of the Florida variety were transformed by means of the biolistic method in accordance with the method described by Becker et al. (1994, Plant Journal 5, 299-307) with a DNA mixture containing the plasmid pML82 and a fragment purified by means of HPLC, which was excised from the plasmid pML8-A.t.-OK1 with the restriction enzymes *AvrII* and *SwaI*, and contains the promoter of the polyubiquitin gene from maize, the total open reading frame of OK1 from *Arabidopsis thaliana* and the *nos*-terminator from *Agrobacterium tumefaciens*. With the help of RT-PCR, plants were identified that have both an expression of the A.t.-Ok1 protein as well as an expression of the S.t.-R1 protein. The obtained plants were designated as TA-R1-OK1.

g) Identification of transgenic wheat plants

T1 plants of the lines TA-R1 and TA-OK1 were cultivated in the greenhouse and sprayed with Basta® (0.5% solution) before blooming. Plants not expressing the Basta® mediated resistance gene died.

h) Manufacture of wheat plants that have an expression of an S.t.-R1 protein and an expression of an A.t.-OK1 protein, by means of crossing

TA-OK1 plants that survived the treatment with Basta® were crossed either with TA-R1 plants that survived the treatment with Basta®, or with homozygotic plants of the line 40A-11-8 described in WO 02 034923. The obtained offspring were designated as TA-Ok1xTA-R1 or as TA-OK1x40A-11-8 respectively.

e) Analysis of the transgenic wheat plants and the starch synthesised from them

Starch was isolated and the content of phosphate covalently bound to the starch was determined for grains resulting from the crossing of lines TA-Ok1 and TA-R1 or TA-OK1 and 40A-11-8 respectively. The phosphate content of starch that was isolated from grains obtained from the crosses TA-Ok1 and TA-R1 or TA-OK1 and 40A-11-8 was significantly higher for some plants than that of starch that was isolated from grains from corresponding wild type plants or from plants of the line 40A-11-8.

Grains of the respective plants were harvested for the analysis of the starch from various TA-R1-OK1 lines, and the C-6 phosphate content and the C-3 phosphate content of the isolated starch was analysed. It was possible to identify some plants for which the content of C-6 phosphate plus C-3 phosphate was clearly higher in comparison with the content of C-6 phosphate plus C-3 phosphate from starch isolated from grains of the lines TA-R1 or 40A-11-8.

14. Manufacture of transgenic rice plants that have an increased expression of an OK1 protein and an R1 protein

a) Manufacture of the plasmid pGlo-A.t.-OK1

The plasmid pIR94 was obtained by amplifying the promoter of the globulin gene from rice by means of a polymerase chain reaction (30 x 20 sec 94 °C, 20 sec 62 °C, 1 min

68 °C, 4 mM Mg2SO4) with the primers glb1-F2 (AAAACAATTGGCGCCTGGAGGGAGGAGA) and glb1-R1 (AAAACAATTGATGATCAATCAGACAATCACTAGAA) on the genomic DNA of rice of the variety M202 with High Fidelity Taq Polymerase (Invitrogen, catalogue number 11304-011) and cloned in pCR2.1 (Invitrogen catalogue number K2020-20).

The plasmid pIR115 was obtained by cloning a synthetic piece of DNA consisting of the two oligonucleotides X1 (TGCAGGCTGCAGAGCTCCTAGGCTCGAGTTAACACTAGTAAGCTTAATTAAGAT ATCATTTAC) and X2 (AATTGTAAATGATATCTTAATTAAGCTTACTAGTGTTAACTCGAGCCTAGGAGCT CTGCAGCCTGCA) in the vector pGSV71 excised with *Sda*I and *Mun*I.

The plasmid pIR115 obtained was excised with *Sda*I, the protruding 3'-ends smoothed with T4 DNA polymerase and a *HindIII / SphI* fragment from pBinAR (Höfgen and Willmitzer, 1990, Plant Science 66, 221-230) with a size of 197 base pairs, smoothed by means of T4 DNA polymerase and containing the termination signal of the octopine synthase gene from *Agrobacterium tumefaciens*, was inserted. The obtained plasmid was designated as pIR96.

The plasmid pIR103 was obtained in which a DNA fragment cloned from pIR94 with a length of 986 base pairs containing the promoter of the globulin gene from rice was cloned in the plasmid pIR96.

pGSV71 is a derivative of the plasmid pGSV7, which derives from the intermediate vector pGSV1. pGSV1 constitutes a derivative of pGSC1700, the construction of which has been described by Cornelissen and Vanderwiele (Nucleic Acid Research 17, (1989), 19-25). pGSV1 was obtained from pGSC1700 by deletion of the carbenicillin resistance gene and deletion of the T-DNA sequences of the TL-DNA region of the plasmid pTiB6S3.

pGSV7 contains the replication origin of the plasmid pBR322 (Bolivar et al., Gene 2, (1977), 95-113) as well as the replication origin of the Pseudomonas plasmid pVS1 (Itoh et al., Plasmid 11, (1984), 206). pGSV7 also contains the selectable marker gene *aadA*, from the transposon Tn1331 from Klebsiella pneumoniae, which gives

resistance against the antibiotics spectinomycin and streptomycin (Tolmasky, Plasmid 24 (3), (1990), 218-226; Tolmasky and Crosa, Plasmid 29(1), (1993), 31-40).

The plasmid pGSV71 was obtained by cloning a chimeric *bar* gene between the border regions of pGSV7. The chimeric *bar* gene contains the promoter sequence of the cauliflower mosaic virus for initiating the transcription (Odell et al., Nature 313, (1985), 180), the *bar* gene from *Streptomyces hygroscopicus* (Thompson et al., Embo J. 6, (1987), 2519-2523) and the 3'-untranslated area of the nopaline synthase gene of the T-DNA of pTiT37 for terminating the transcription and polyadenylation. The *bar* gene provides tolerance against the herbicide glufosinate ammonium.

A DNA fragment, which contains the sequence of the total open reading frame of the OK1 protein from *Arabidopsis*, was excised from the vector A.t.-OK1-pGEM and cloned in the vector pIR103. For this purpose, the plasmid A.t.-OK1-pGEM was excised with the restriction enzymes *Bsp*120I, the ends smoothed with T4-DNA polymerase and excised with *Sac*I. The DNA fragment coding the OK1 protein from *Arabidopsis thaliana* was cloned in the vector pIR103 excised with *Ecl*136II and *Xho*I. The obtained plasmid was designated as pGlo-A.t.-OK1.

b) Transformation of rice plants with the plasmid pGlo-A.t.-OK1

Rice plants (variety M202) were transformed by means of *Agrobacterium* (containing the plasmid pGlo-A.t.-OK1) using the method described by Hiei et al. (1994, Plant Journal 6(2), 271-282).

c) Analysis of the transgenic rice plants that were transformed with the plasmid pGlo-A.t.-OK1

By means of quantitative RT PCR analysis, it was possible to identify plants that had an expression of mRNA coding the A.t.-OK1 protein. Homozygotic plants of the T1 generation were identified as described above in Example 11. g) by means of maize plants. The obtained plants were designated as OS-OK1.

d) Transformation of rice plants with the plasmid pML82

Rice plants (variety M202) were transformed by means of Agrobacterium (containing the plasmid pML82) using the method described by Hiei et al. (1994, Plant Journal 6(2), 271-282).

e) Analysis of the transgenic rice plants that were transformed with the plasmid pGloML82

By means of quantitative RT PCR analysis, it was possible to identify plants that had an expression of mRNA coding the S.t.-R1 protein. Homozygotic plants of the T1 generation were identified as described above in Example 11. g) by means of maize plants. The obtained plants were designated as OS-R1.

f) Manufacture of rice plants that have an expression of an S.t.-R1 protein and an expression of an A.t.-OK1 protein, by means of crossing

Homozygotic OS-OK1 plants were crossed with homozygotic OS-R1 plants. The obtained plasmid was designated as OS-Ok1xOS-R1.

g) Analysis of the transgenic rice plants and the starch synthesised by them

Starch was isolated and the content of phosphate covalently bound to the starch was determined for grains resulting from the crosses of OS-Ok1xOS-R1. The phosphate content in the C-6 position and in the C-3 position of the glucose molecules of starch that was isolated from grains obtained from crosses of the lines OS-Ok1 and OS-R1 was for some lines clearly higher than for starch that was isolated from grains from corresponding wild type plants or from plants of the line OS-R1.